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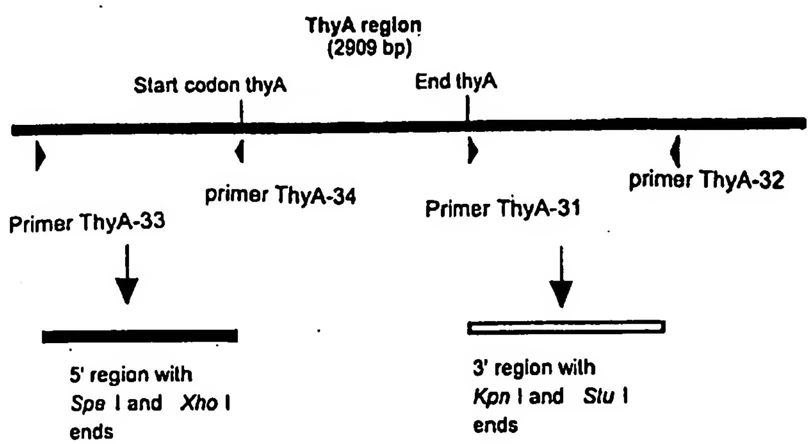
(54) Title: METHOD OF PRODUCING *THY* A⁻STRAINS OF *VIBRIO CHOLERAE*, SUCH STRAINS AND THEIR USE



(57) Abstract

A method of producing a *thy* A⁻strain of *vibrio cholerae* comprising the step of site-directed mutagenesis in the *V. cholerae* chromosome at the locus of the *thy* A gene SEQ ID NO: 1 of FIG. 1, is described. Particularly, a Δ *thy* A strain of *Vibrio cholerae* lacking the functionality of the *thy* A is disclosed. This strain may comprise one or several episomal autonomously replicating DNA elements, such as plasmids, having an optionally foreign, e.g. *E. coli*, functional *thy* A gene that enables the strain to grow in the absence of thymine in the growth medium, and optionally having a structural gene encoding a homologous or heterologous protein. Further, proteins encoded by a structural *thy* A gene and the 5'-flanking region are described as SEQ ID NO: 4 of FIG. 4 and SEQ ID NO: 5 of FIG. 5, respectively. Additionally, a vaccine comprising a *Vibrio cholerae* Δ *thy* A strain of the invention or a *thy* A⁻strain of *Vibrio cholerae* produced by the method of the invention is disclosed.

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FIG. 1

SEQ ID NO: 1:

GAGAAGGTTT GTTATGCCTC AGGGTTATCT GCAGTTTCCC AATATTGACC CCGTATTGTT 60

TTCGATCGGC CCTCTAGCGG TGCGCTGGTA TGGCTTGATG TATTTGGTGG GTTTCCTTTT 120

TGCTATGTGG TTGGCCAATC GCCGAGCGGA TCGCGCGGGC AGTGGTTGGA CGCGTGAGCA 180

AGTCTCTGAC TTGTTATTCG CCGGCTTTTT AGGTGTAGTG ATCGGTGGCC GAGTTGGTTA 240

TGTGATCTTC TACAATTTTG ATCTGTTCCT TGCTGACCCT CTTTATTTAT TCAAAGTGTG 300

GACTGGCGGC ATGTCCTTCC ACGGCGGCTT ATTGGGTGTG ATCACCGCCA TGTTCTGGTA 360

TGCGCGTAAA AACCAACGCA CCTTCTTTGG TGTGGCCGAT TTTGTTGCCC CTTTAGTGCC 420

ATTCGGTTTG GGGATGGGAC GTATCGGTAA CTTTATGAAT AGTGAACTTT GGGGACGAGT 480

AACGGATGTG CCTTGGGCTT TTGTATTCCC TAATGGTGGC CCACTGCCGC GCCATCCTTC 540

ACAGCTTTAT GAATTCGCCT TAGAAGGCGT GGTTCTGTTC TTTATTCTTA ATTGGTTTAT 600

TGGTAAACCT CGTCCGCTAG GCAGCGTATC CGGACTGTTT TTAGCTGGAT ACGGTACATT 660

CCGCTTCCTT GTGGAATACG TCCGTGAGCC AGATGCTCAG TTGGGTCTGT TTGGTGGCTT 720

CATTTCAATG GGGCAAATCC TCTCCTTACC TATGGTGATC ATCGGTATTT TGATGATGGT 780

TTGGTCTTAC AAGCGCGGTT TGTATCAAGA CCGTGTAGCA GCAAAATAGG GTAGTTAGGT 840

GAAACAGTAT TTAGATCTTT GTCAGCGCAT CGTCGATCAA GGTGTTTGGG TTGAAAATGA 900

ACGAACGGGC AAGCGTTGTT TGACTGTGAT TAATGCCGAT TTGACCTACG ATGTGGGCAA 960

CAATCAGTTT CCTCTAGTGA CTACACGCAA GAGTTTTTGG AAAGCTGCCG TAGCCGAGTT 1020

GCTCGGCTAT ATTCGTGGTT ACGATAATGC GGCGGATTTT CGCCAATTAG GTACCAAAAC 1080

CTGGGATGCT AATGCCAATT TAAACCAAGC ATGGCTCAAC AATCCTTACC GTAAAGGTGA 1140

GGATGACATG GGACGCGTGT ATGGTGTTCA GGGTAGAGCT TGGGCTAAGC CTGATGGTGG 1200

TCATATTGAC CAGTTGAAAA AGATTGTTGA TGATTTGAGC CGTGGCGTTG ATGACCGAGG 1260

TGAAATTCTT AACTTCTACA ATCCGGGTGA ATTTCACATG GGGTGTTTGC GCCCTTGCAT 1320

GTACAGCCAT CATTTTTCAT TGCTGGGGGA TACCTTGTAT CTCAACAGTA CTCAGCGTTC 1380

ATGTGATGTG CCCTTGGGGT TGAATTTCAA CATGGTGCAG GTTTATGTGT TCCTTGCGCT 1440

GATGGCACAG ATCACAGGGA AAAAGCCGGG CTTGGCGTAT CACAAGATCG TCAATGCGCA 1500

FIG.1 (cont.)

```
CATTTACCAA GATCAACTCG AATTGATGCG CGATGTGCAG CTAAAACGTG AGCCATTCCC 1560
AGCGCCTCAG TTCCATATCA ATCCAAAGAT TAAAACACTG CAGGATTTGG AAACTTGGGT 1620
CACTTTGGAT GATTTTGACG TCACCGGATA TCAGTTCCAC GATCCTATTC AATACCCGTT 1680
TTCAGTCTAA TCCCGTATTC AGGCGGTATG GCTTGATGGG TTTTATATAA AAAAAGCTCC 1740
CGAAGGTCGG GAGCTTTTTT TATACAGATG ATGCTTTAAC GCTTAAGCGG TTAGGGCAAG 1800
AATGCTGCCG GGGATGACGA CAAACACACC CAATAAGTAA CTCACCACCA CCATTTTGCT 1860
CTTACAAGCC CAAGTTGAGA TGAGCTCAGC ACCTTTAATA GGCAGTTCGC GTAAGAAAGG 1920
AATACCGTAA ATCAAGACCG TAGCCATCAA GTTAAAGCTT AAGTGCACCA GCGCAATTTG 1980
CAGAGCAAAC ACGGCAAACT CACCAGAGAC AGCGGTTGCG GCGAGCAGAG CAGTAATACA 2040
AGTGCCAATG TTCGCACCTA AGGTAAATGG GTAGATTTCA CGCACTTTCA GCACGCCAGA 2100
GCCCACGAGA GGAACCATTA GGCTGGTTGT GGTCGATGAA GATTGAACTA ATACCGTAAC 2160
CACTGTACCT GAAGCAATAC CGTGTAGTGG GCCTCGGCCA ATCGCATTTT GTAGAATTTC 2220
ACGTGCGCGG CCAACCATCA AACTCTTCAT CAGTTTGCCC ATCACCGTAA TGGCGACGAA 2280
AATGGTCGCA ATACCCAATA CGATAAGTGC GACACCACCG AAAGTATTAC CCAATACCGA 2340
AAGCTGGGTT TCAAGCCCTG TGATGACAGG TTTGGTAATC GGTTTGATAA AATCAAAACC 2400
TTTCATGCTC ATATCGCCAG TCGCAAGCAG AGGCGAAACG AGCCAGTGTG AGACTTTCTC 2460
TAAAATGCCA AACATCATTT CTAGAGGTAG GAAGATCAGC ACCGCGAGAA GATTGAAAAA 2520
ATCGTGGATG GTGGCACTGG CGAAAGCACG GCGAAACTCT TCTTTACAGC GCATATGGCC 2580
AAGGCTGACG AGAGTATTGG TCACAGTAGT ACCAATATTG GCACCCATCA CCATAGGAAT 2640
CGCGGTTTCA ACCGGTAACC CACCGGCAAC GAGACCAACA ATAATAGAAG TCACCGTGCT 2700
TGAGGATTGA ATCAGTGCCG TTGCCACTAA ACCAATCATC AATCCTGCAA TTGGGTGGGA 2760
AGCAAATTCA AATAGAACTT TGGCTTGATC GCCGGTTGCC CATTTAAAAC CGCTGCCGAC 2820
CATCGCGACT GCAAGAAGTA GTAAATACAG CATGAAAGCC AAGTTTGCCC AACGTAGGCC 2880
TTTCGTGGTC AGCGAAATCG GCGCTGCAG                                   2909
```

FIG.2

SEQ ID NO: 2:

```
GAGAAGGTTT GTTATGCCTC AGGGTTATCT GCAGTTTCCC AATATTGACC CCGTATTGTT   60
TTCGATCGGC CCTCTAGCGG TGCGCTGGTA TGGCTTGATG TATTTGGTGG GTTTCCTTTT  120
TGCTATGTGG TTGGCCAATC GCCGAGCGGA TCGCGCGGGC AGTGGTTGGA CGCGTGAGCA  180
AGTCTCTGAC TTGTTATTCG CCGGCTTTTT AGGTGTAGTG ATCGGTGGCC GAGTTGGTTA  240
TGTGATCTTC TACAATTTTG ATCTGTTCCT TGCTGACCCT CTTTATTTAT TCAAAGTGTG  300
GACTGGCGGC ATGTCCTTCC ACGGCGGCTT ATTGGGTGTG ATCACCGCCA TGTTCTGGTA  360
TGCGCGTAAA AACCAACGCA CCTTCTTTGG TGTGGCCGAT TTTGTTGCCC CTTTAGTGCC  420
ATTCGGTTTG GGGATGGGAC GTATCGGTAA CTTTATGAAT AGTGAACTTT GGGGACGAGT  480
AACGGATGTG CCTTGGGCTT TTGTATTCCC TAATGGTGGC CCACTGCCGC GCCATCCTTC  540
ACAGCTTTAT GAATTCGCCT TAGAAGGCGT GGTTCTGTTC TTTATTCTTA ATTGGTTTAT  600
TGGTAAACCT CGTCCGCTAG GCAGCGTATC CGGACTGTTT TTAGCTGGAT ACGGTACATT  660
CCGCTTCCTT GTGGAATACG TCCGTGAGCC AGATGCTCAG TTGGGTCTGT TTGGTGGCTT  720
CATTTCAATG GGGCAAATCC TCTCCTTACC TATGGTGATC ATCGGTATTT TGATGATGGT  780
TTGGTCTTAC AAGCGCGGTT TGTATCAAGA CCGTGTAGCA GCAAAATAGG GTAGTTAG    838
```

FIG.3

SEQ ID NO: 3:

```
TAATCCCGTA TTCAGGCGGT ATGGCTTGAT GGGTTTTATA TAAAAAAAGC TCCCGAAGGT   60
CGGGAGCTTT TTTTATACAG ATGATGCTTT AACGCTTAAG CGGTTAGGGC AAGAATGCTG  120
CCGGGGATGA CGACAAACAC ACCCAATAAG TAACTCACCA CCACCATTTT GCTCTTACAA  180
GCCCAAGTTG AGATGAGCTC AGCACCTTTA ATAGGCAGTT CGCGTAAGAA AGGAATACCG  240
TAAATCAAGA CCGTAGCCAT CAAGTTAAAG CTTAAGTGCA CCAGCGCAAT TTGCAGAGCA  300
AACACGGCAA ACTCACCAGA GACAGCGGTT GCGGCGAGCA GAGCAGTAAT ACAAGTGCCA  360
ATGTTCGCAC CTAAGGTAAA TGGGTAGATT TCACGCACTT TCAGCACGCC AGAGCCCACG  420
AGAGGAACCA TTAGGCTGGT TGTGGTCGAT GAAGATTGAA CTAATACCGT AACCACTGTA  480
CCTGAAGCAA TACCGTGTAG TGGGCCTCGG CCAATCGCAT TTTGTAGAAT TTCACGTGCG  540
CGGCCAACCA TCAAACTCTT CATCAGTTTG CCCATCACCG TAATGGCGAC GAAAATGGTC  600
GCAATACCCA ATACGATAAG TGCGACACCA CCGAAAGTAT TACCCAATAC CGAAAGCTGG  660
GTTTCAAGCC CTGTGATGAC AGGTTTGGTA ATCGGTTTGA TAAAATCAAA ACCTTTCATG  720
CTCATATCGC CAGTCGCAAG CAGAGGCGAA ACGAGCCAGT GTGAGACTTT CTCTAAAATG  780
CCAAACATCA TTTCTAGAGG TAGGAAGATC AGCACCGCGA GAAGATTGAA AAAATCGTGG  840
ATGGTGGCAC TGGCGAAAGC ACGGCGAAAC TCTTCTTTAC AGCGCATATG GCCAAGGCTG  900
ACGAGAGTAT TGGTCACAGT AGTACCAATA TTGGCACCCA TCACCATAGG AATCGCGGTT  960
TCAACCGGTA ACCCACCGGC AACGAGACCA ACAATAATAG AAGTCACCGT GCTTGAGGAT 1020
TGAATCAGTG CCGTTGCCAC TAAACCAATC ATCAATCCTG CAATTGGGTG GGAAGCAAAT 1080
TCAAATAGAA CTTTGGCTTG ATCGCCGGTT GCCCATTTAA AACCGCTGCC GACCATCGCG 1140
ACTGCAAGAA GTAGTAAATA CAGCATGAAA GCCAAGTTTG CCCAACGTAG GCCTTTCGTG 1200
GTCAGCGAAA TCGGCGCTGC AG                                          1222
```

FIG.4

SEQ ID NO: 4:

```
Val Lys Gln Tyr Leu Asp Leu Cys Gln Arg Ile Val Asp Gln Gly Val
1               5                   10                  15

Trp Val Glu Asn Glu Arg Thr Gly Lys Arg Cys Leu Thr Val Ile Asn
            20                  25                  30

Ala Asp Leu Thr Tyr Asp Val Gly Asn Asn Gln Phe Pro Leu Val Thr
        35                  40                  45

Thr Arg Lys Ser Phe Trp Lys Ala Ala Val Ala Glu Leu Leu Gly Tyr
    50                  55                  60

Ile Arg Gly Tyr Asp Asn Ala Ala Asp Phe Arg Gln Leu Gly Thr Lys
65                  70                  75                  80

Thr Trp Asp Ala Asn Ala Asn Leu Asn Gln Ala Trp Leu Asn Asn Pro
                85                  90                  95

Tyr Arg Lys Gly Glu Asp Asp Met Gly Arg Val Tyr Gly Val Gln Gly
            100                 105                 110

Arg Ala Trp Ala Lys Pro Asp Gly Gly His Ile Asp Gln Leu Lys Lys
        115                 120                 125

Ile Val Asp Asp Leu Ser Arg Gly Val Asp Asp Arg Gly Glu Ile Leu
    130                 135                 140

Asn Phe Tyr Asn Pro Gly Glu Phe His Met Gly Cys Leu Arg Pro Cys
145                 150                 155                 160

Met Tyr Ser His His Phe Ser Leu Leu Gly Asp Thr Leu Tyr Leu Asn
                165                 170                 175

Ser Thr Gln Arg Ser Cys Asp Val Pro Leu Gly Leu Asn Phe Asn Met
            180                 185                 190

Val Gln Val Tyr Val Phe Leu Ala Leu Met Ala Gln Ile Thr Gly Lys
        195                 200                 205

Lys Pro Gly Leu Ala Tyr His Lys Ile Val Asn Ala His Ile Tyr Gln
    210                 215                 220

Asp Gln Leu Glu Leu Met Arg Asp Val Gln Leu Lys Arg Glu Pro Phe
225                 230                 235                 240

Pro Ala Pro Gln Phe His Ile Asn Pro Lys Ile Lys Thr Leu Gln Asp
                245                 250                 255

Leu Glu Thr Trp Val Thr Leu Asp Asp Phe Asp Val Thr Gly Tyr Gln
            260                 265                 270

Phe His Asp Pro Ile Gln Tyr Pro Phe Ser Val
        275                 280
```

FIG. 5

SEQ ID NO: 5:

Met Pro Gln Gly Tyr Leu Gln Phe Pro Asn Ile Asp Pro Val Leu Phe
1 5 10 15

Ser Ile Gly Pro Leu Ala Val Arg Trp Tyr Gly Leu Met Tyr Leu Val
20 25 30

Gly Phe Leu Phe Ala Met Trp Leu Ala Asn Arg Arg Ala Asp Arg Ala
35 40 45

Gly Ser Gly Trp Thr Arg Glu Gln Val Ser Asp Leu Leu Phe Ala Gly
50 55 60

Phe Leu Gly Val Val Ile Gly Gly Arg Val Gly Tyr Val Ile Phe Tyr
65 70 75 80

Asn Phe Asp Leu Phe Leu Ala Asp Pro Leu Tyr Leu Phe Lys Val Trp
85 90 95

Thr Gly Gly Met Ser Phe His Gly Gly Leu Leu Gly Val Ile Thr Ala
100 105 110

Met Phe Trp Tyr Ala Arg Lys Asn Gln Arg Thr Phe Phe Gly Val Ala
115 120 125

Asp Phe Val Ala Pro Leu Val Pro Phe Gly Leu Gly Met Gly Arg Ile
130 135 140

Gly Asn Phe Met Asn Ser Glu Leu Trp Gly Arg Val Thr Asp Val Pro
145 150 155 160

Trp Ala Phe Val Phe Pro Asn Gly Gly Pro Leu Pro Arg His Pro Ser
165 170 175

Gln Leu Tyr Glu Phe Ala Leu Glu Gly Val Val Leu Phe Phe Ile Leu
180 185 190

Asn Trp Phe Ile Gly Lys Pro Arg Pro Leu Gly Ser Val Ser Gly Leu
195 200 205

Phe Leu Ala Gly Tyr Gly Thr Phe Arg Phe Leu Val Glu Tyr Val Arg
210 215 220

Glu Pro Asp Ala Gln Leu Gly Leu Phe Gly Gly Phe Ile Ser Met Gly
225 230 235 240

Gln Ile Leu Ser Leu Pro Met Val Ile Ile Gly Ile Leu Met Met Val
245 250 255

Trp Ser Tyr Lys Arg Gly Leu Tyr Gln Asp Arg Val Ala Ala Lys
260 265 270

FIG. 6

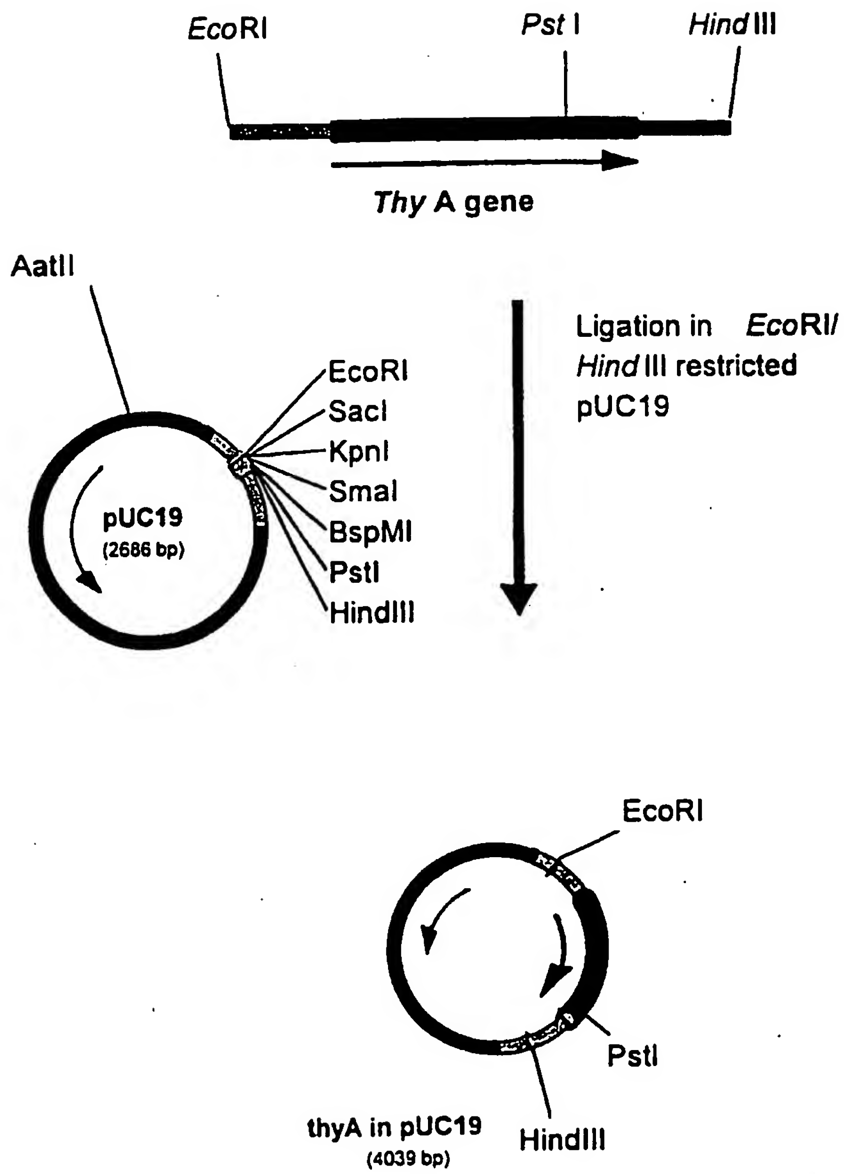

EcoRI
Pst I
Hind III
Thy A gene
AatII
EcoRI
SacI
KpnI
SmaI
BspMI
PstI
HindIII
pUC19
(2686 bp)
Ligation in EcoRI/
Hind III restricted
pUC19
EcoRI
PstI
HindIII
thyA in pUC19
(4039 bp)

FIG. 7

```
E. coli      : MKQYLELMQKVLDEGT-QKNDRTGTGTLSIFGHQMRFNL-QDGFPLVTTKRCHLRSIIHE
V. cholerae  : VKQYLDLCQRIVDQGVWVENERTGKRCLTVINADLTYDVGNNQFPLVTTRKSFWKAAVAE
H. influenza : MKQYLELCRRIVSEGEWVANERTGKHCLTVINADLEYDVANNQFPLITTRKSYWKAAIAE

E. coli      : LLWFLQGDTNIAYHENNVTIWD--------EWADE-----NGDLGPVYGKQWRAWPTPDG
V. cholerae  : LLGYIRGYDNAADFRQLGTKTWDANANLNQAWLNNPYRKGEDDMGRVYGVQGRAWAKPDG
H. influenza : FLGYIRGYDNAADFRALGTKTWDANANENAAWLANPHRRGVDDMGRVYGVQGRAWRKPNG

E. coli      : RHIDQITTVLNQLKNDPDSRRIIVSAWNVGELDKMALAPCHAFFQFYVADGKLSCQLYQR
V. cholerae  : GHIDQLKKIVDDLSRGVDDRGEILNFYNPGEFHMGCLRPCMYSHHFSLLGDTLYLNSTQR
H. influenza : ETIDQLRKIVNNLTKGIDDRGEILTFFNPGEFDLGCLRPCMHTHTFSLVGDTLHLTSYQR

E. coli      : SCDVFLGLPFNIASYALLVHMMAQQCDLEVGDFVWTGGDTHLYSNHMD-QTHLQLSREPR
V. cholerae  : SCDVPLGLNFNMVQVYVFLALMAQITGKKPGLAYHKIVNAHIYQDQLELMRDVQLKREPF
H. influenza : SCDVPLGLNFNQIQVFTFLALMAQITGKKAGKAYHKIVNAHIYEDQLELMRDVQLKREPF

E. coli      : PLPKLIIKRKPESIFDY----RFEDFEIEGYDPHPGIKAPVAI
V. cholerae  : PAPQFHINPKIKTLQDLETWVTLDDFDVTGYQFHDPIQYPFSV
H. influenza : PLPKELINPDIKTLEDLETWVTMDDFKVVGYQSHEPIKYPFSV
```

FIG. 8

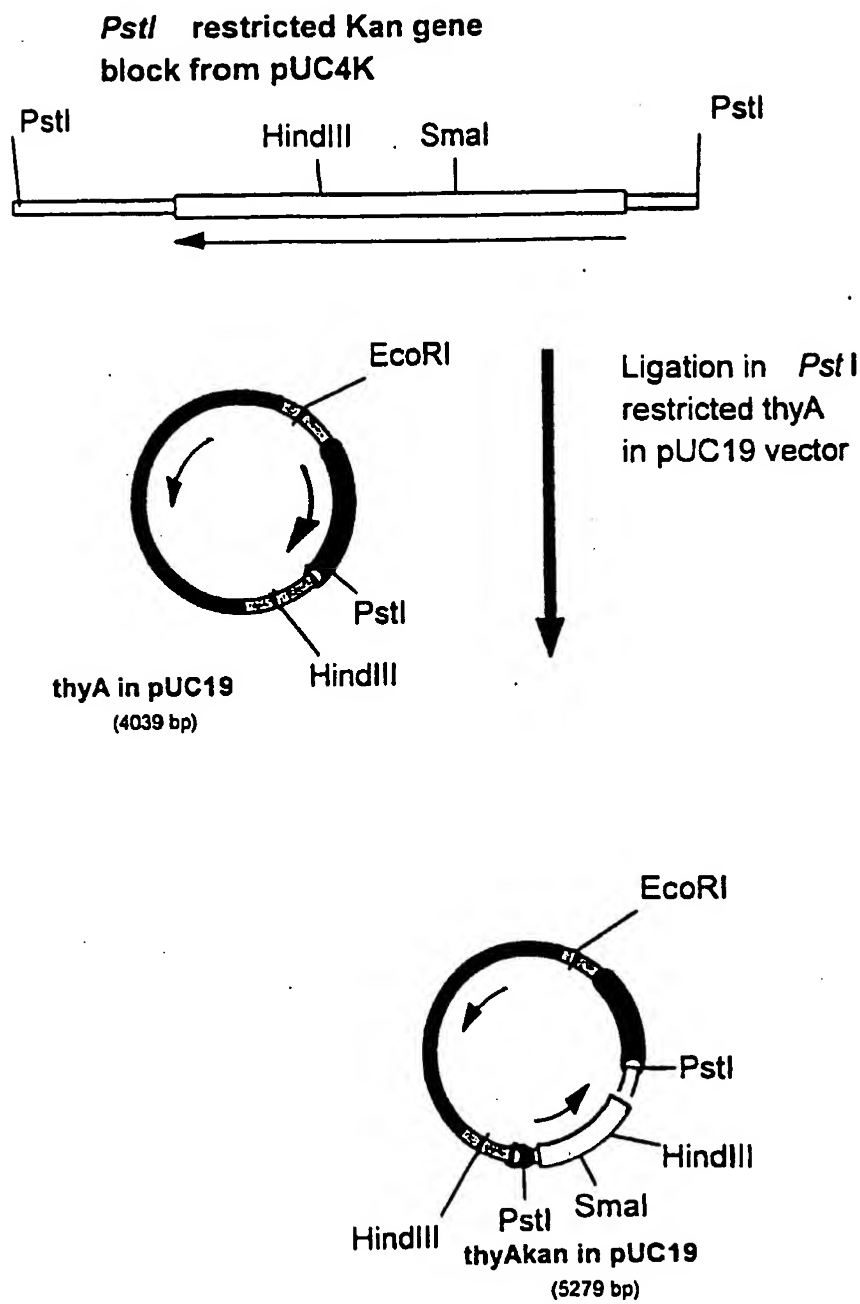
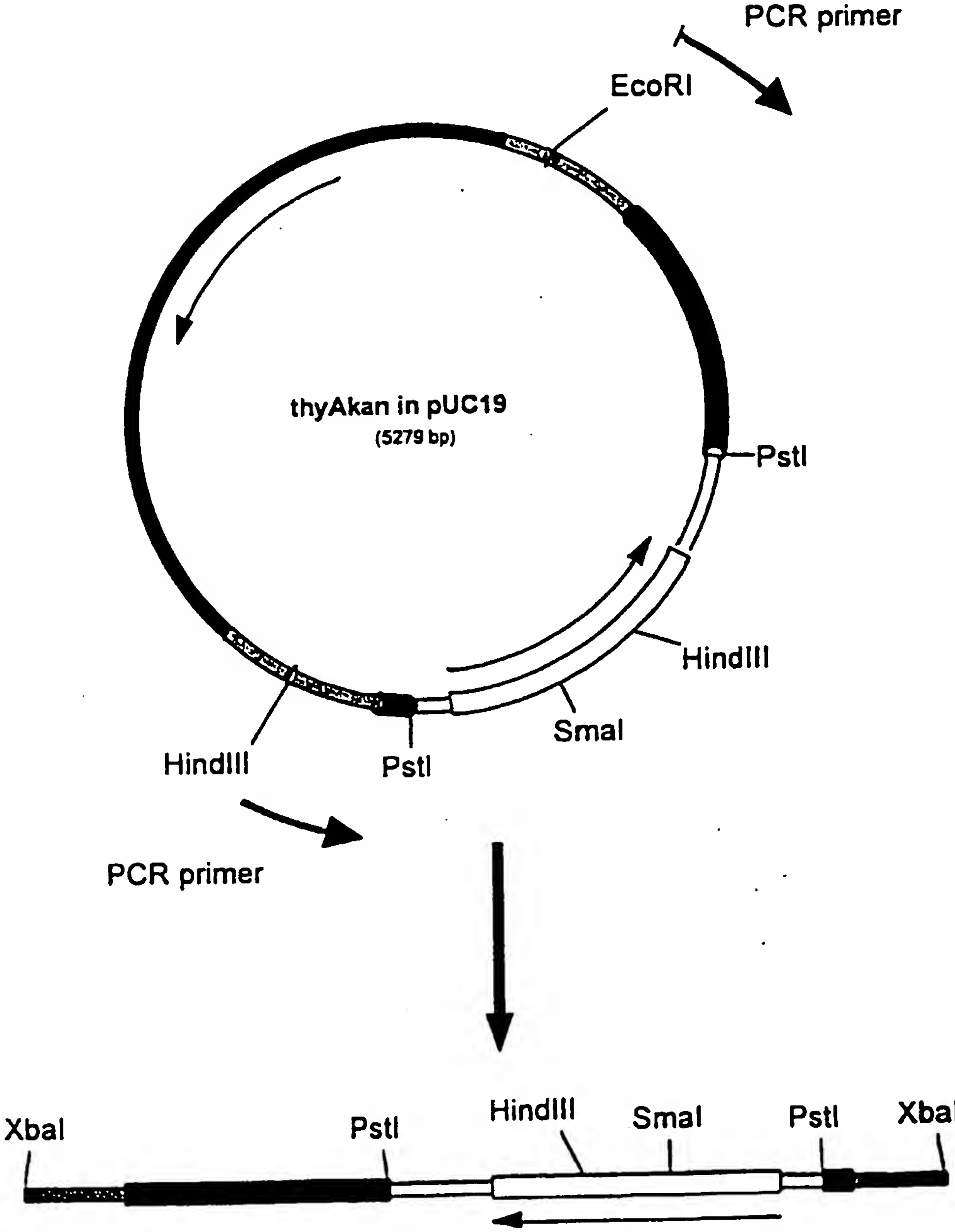

PstI restricted Kan gene
block from pUC4K
PstI
HindIII
SmaI
PstI
EcoRI
Ligation in PstI
restricted thyA
in pUC19 vector
PstI
HindIII
thyA in pUC19
(4039 bp)
EcoRI
PstI
HindIII
SmaI
PstI
HindIII
thyAkan in pUC19
(5279 bp)

FIG. 9

PCR to generate thyA-Kan-thyA fragment with *Xba*I ends. Primers were choosen so that the *Eco*RI and *Hind*III sites were eliminated



thyA with kan XbaI ends
(2643 bp)

FIG. 10

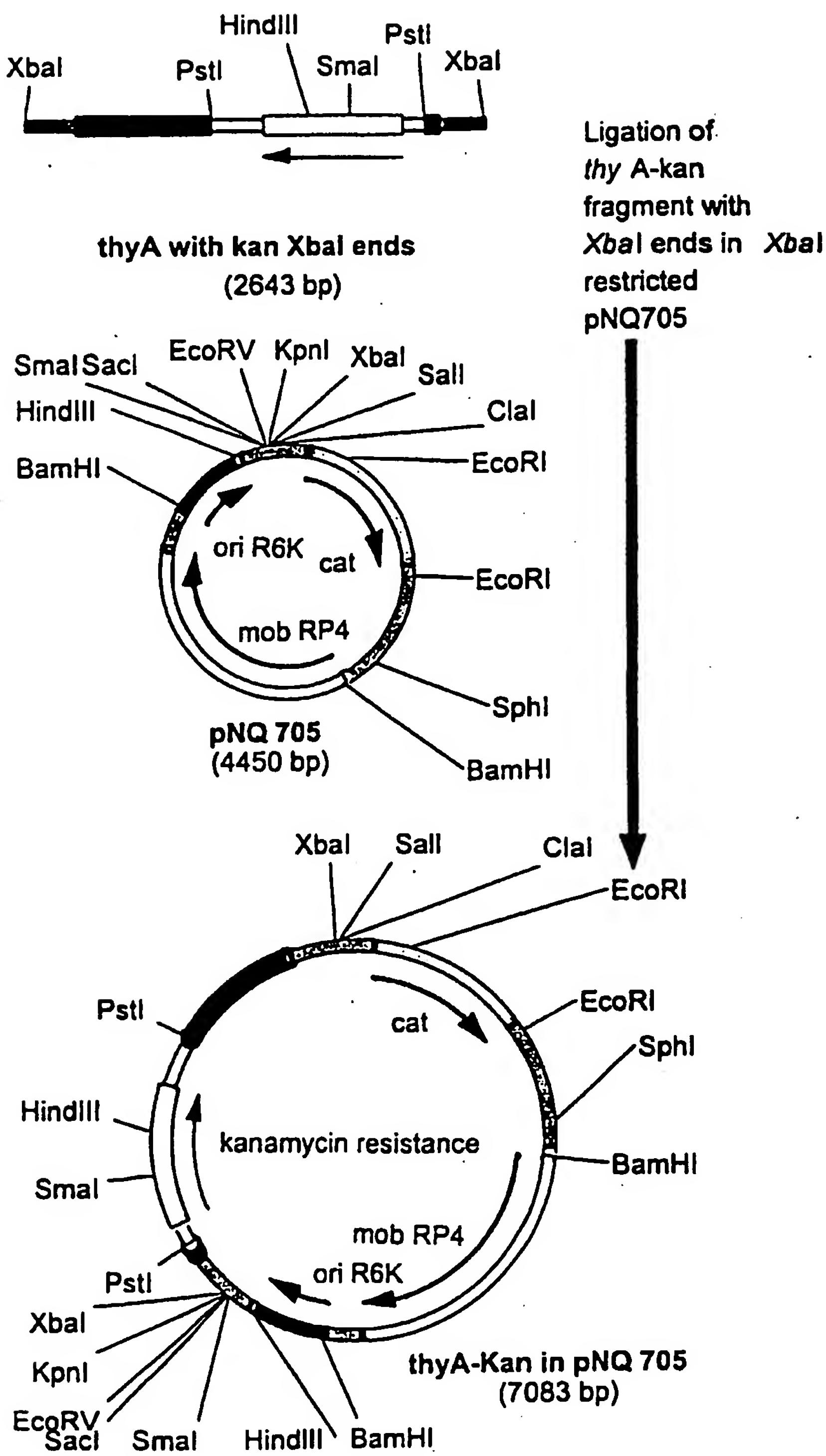

HindIII
PstI
XbaI
PstI
SmaI
XbaI
thyA with kan XbaI ends
(2643 bp)
Ligation of
thy A-kan
fragment with
XbaI ends in XbaI
restricted
pNQ705
SmaI SacI
EcoRV
KpnI
XbaI
SalI
HindIII
ClaI
BamHI
EcoRI
ori R6K
cat
EcoRI
mob RP4
SphI
pNQ 705
(4450 bp)
BamHI
XbaI
SalI
ClaI
EcoRI
EcoRI
PstI
cat
SphI
HindIII
kanamycin resistance
BamHI
SmaI
mob RP4
ori R6K
PstI
XbaI
KpnI
thyA-Kan in pNQ 705
(7083 bp)
EcoRV
SacI
SmaI
HindIII
BamHI

FIG. 11

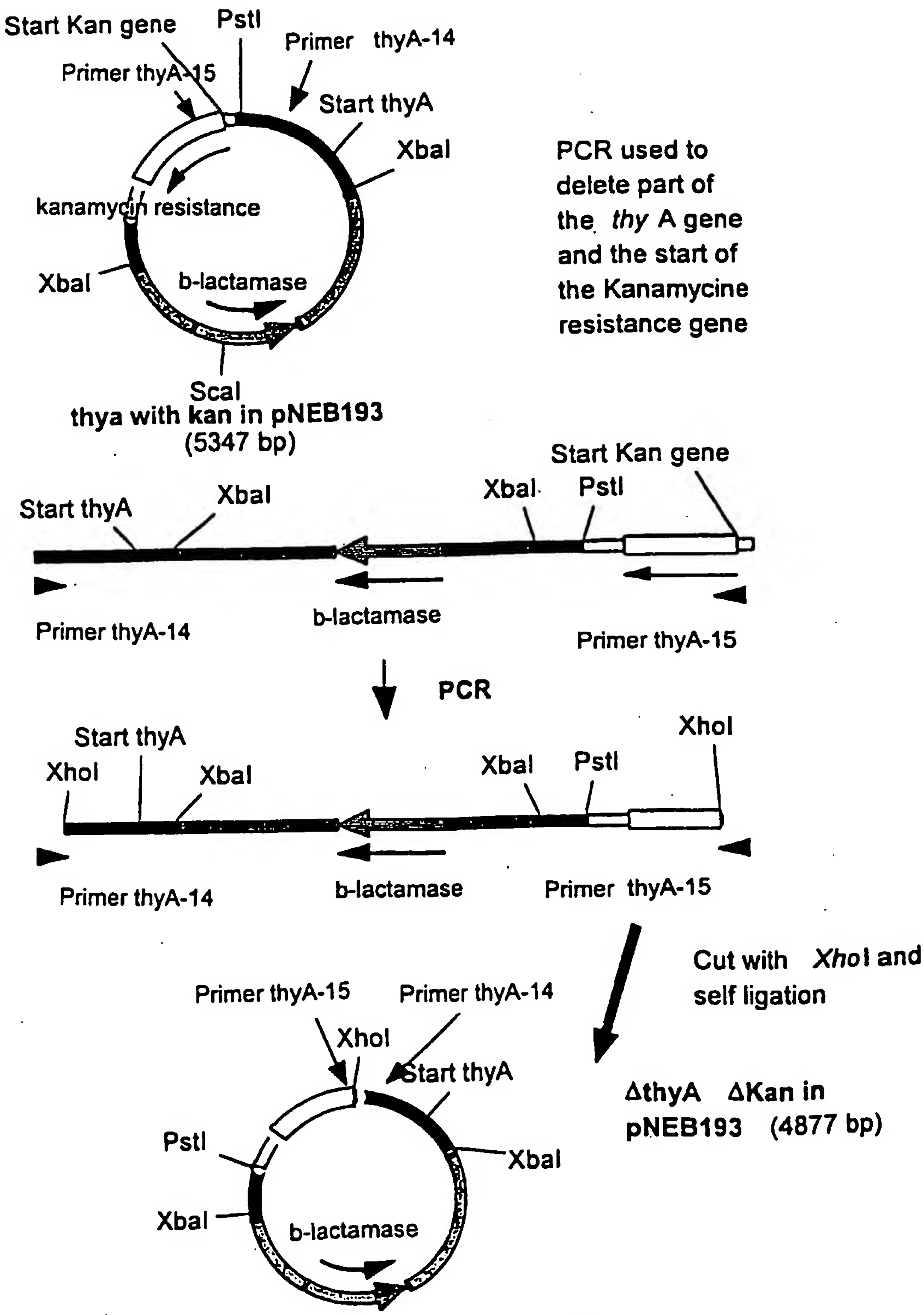

Start Kan gene
PstI
Primer thyA-14
Primer thyA-15
Start thyA
XbaI
kanamycin resistance
XbaI
b-lactamase
ScaI
thya with kan in pNEB193
(5347 bp)
PCR used to delete part of the *thy* A gene and the start of the Kanamycine resistance gene
Start Kan gene
XbaI
PstI
Start thyA
XbaI
Primer thyA-14
b-lactamase
Primer thyA-15
PCR
XhoI
Start thyA
XhoI
XbaI
XbaI
PstI
Primer thyA-14
b-lactamase
Primer thyA-15
Cut with *Xho*I and self ligation
Primer thyA-15
Primer thyA-14
XhoI
Start thyA
PstI
XbaI
XbaI
b-lactamase
ΔthyA ΔKan in pNEB193 (4877 bp)

FIG. 12

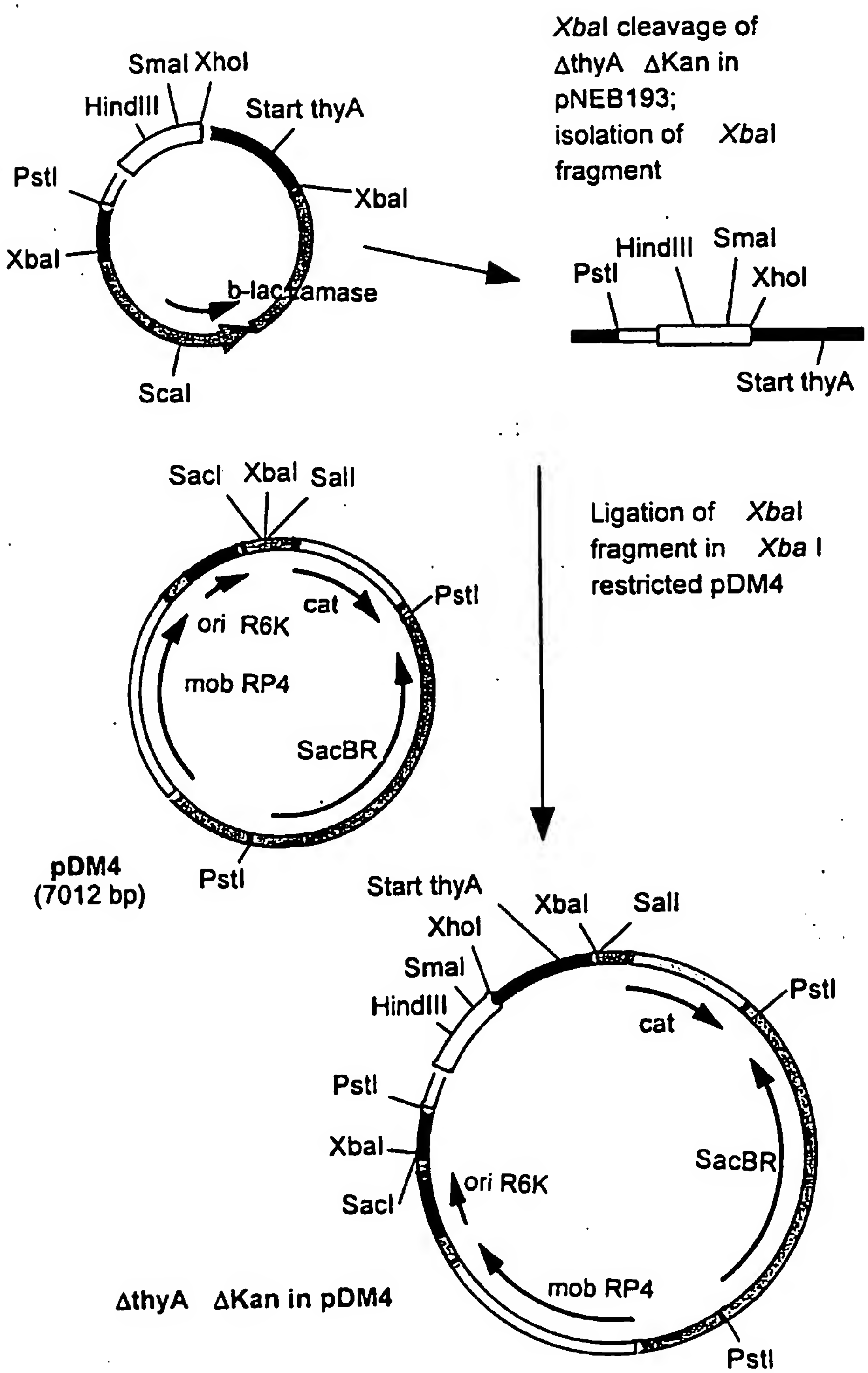

SmaI XhoI
HindIII
Start thyA
PstI
XbaI
XbaI
b-lactamase
ScaI
*Xba*I cleavage of ΔthyA ΔKan in pNEB193; isolation of *Xba*I fragment
HindIII
SmaI
PstI
XhoI
Start thyA
SacI
XbaI
SalI
cat
PstI
ori R6K
mob RP4
SacBR
pDM4
(7012 bp)
PstI
Ligation of *Xba*I fragment in *Xba* I restricted pDM4
Start thyA
XbaI
SalI
XhoI
SmaI
PstI
HindIII
cat
PstI
XbaI
SacBR
SacI
ori R6K
mob RP4
ΔthyA ΔKan in pDM4
PstI

FIG. 13

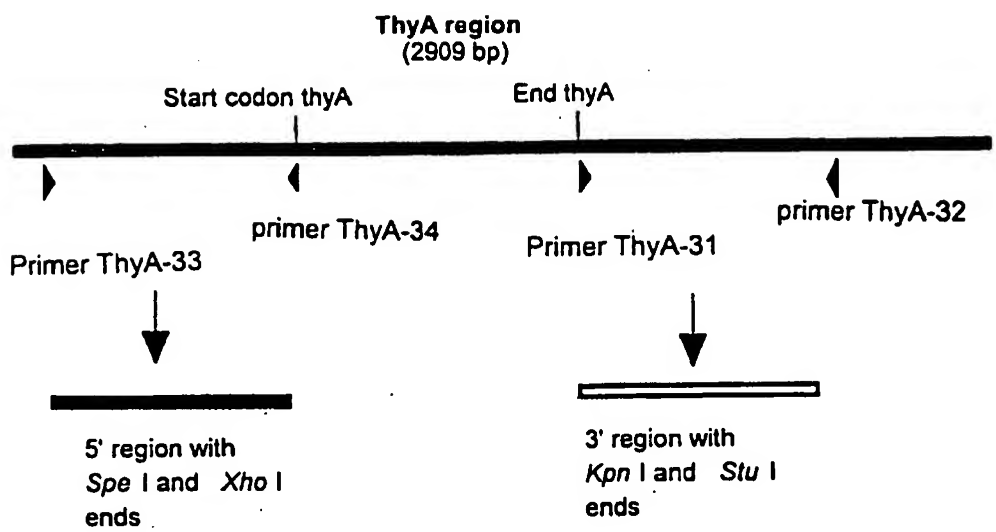

ThyA region
(2909 bp)
Start codon thyA
End thyA
primer ThyA-34
primer ThyA-32
Primer ThyA-33
Primer ThyA-31
5' region with
Spe I and *Xho* I
ends
3' region with
Kpn I and *Stu* I
ends

FIG. 14

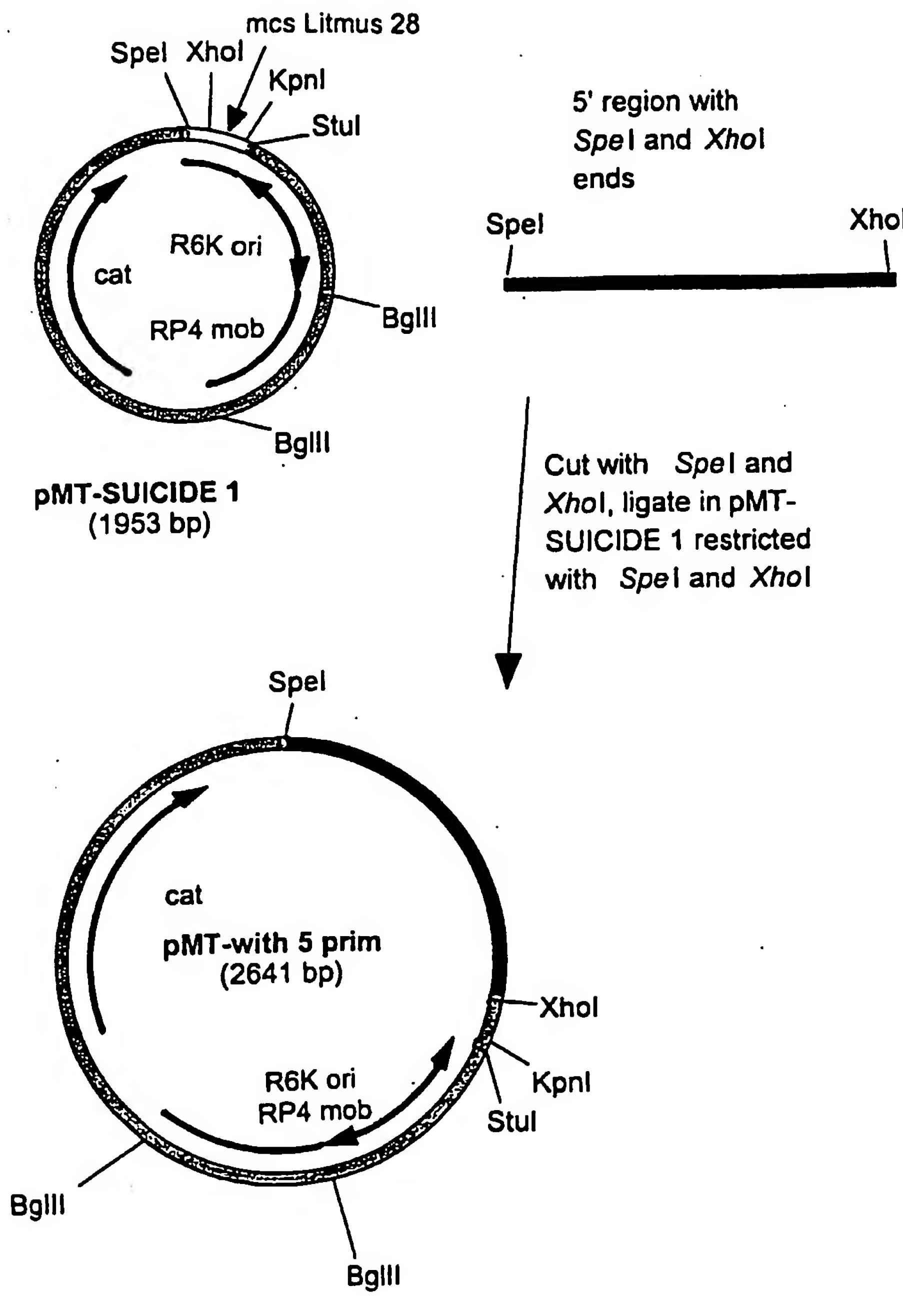

mcs Litmus 28
SpeI
XhoI
KpnI
StuI
R6K ori
cat
RP4 mob
BglII
BglII
pMT-SUICIDE 1
(1953 bp)
5' region with
*Spe*I and *Xho*I
ends
SpeI
XhoI
Cut with *Spe*I and *Xho*I, ligate in pMT-SUICIDE 1 restricted with *Spe*I and *Xho*I
SpeI
cat
pMT-with 5 prim
(2641 bp)
XhoI
KpnI
StuI
R6K ori
RP4 mob
BglII
BglII

FIG. 15

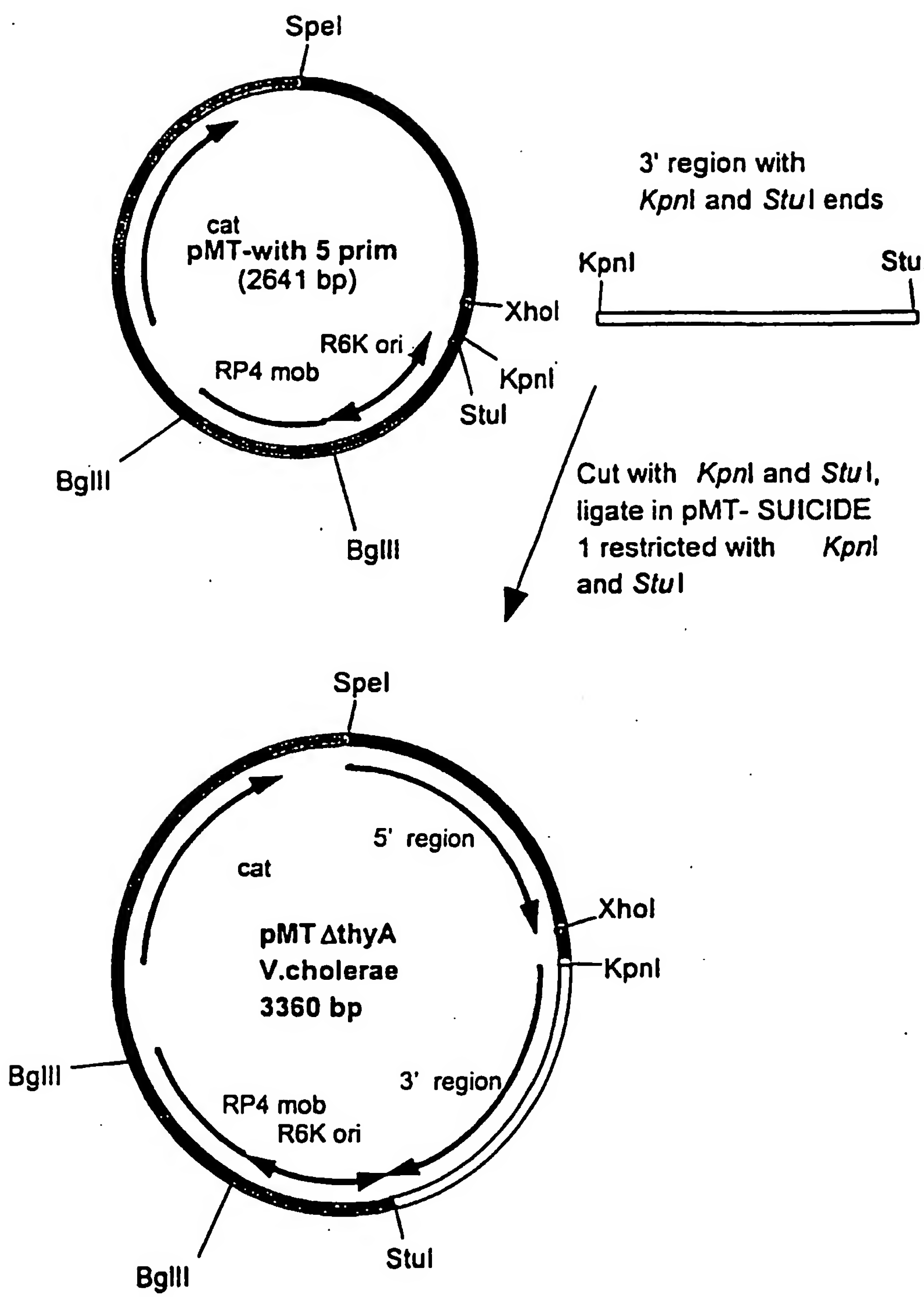

SpeI
cat
pMT-with 5 prim
(2641 bp)
R6K ori
RP4 mob
XhoI
KpnI
StuI
BglII
BglII
3' region with
KpnI and *StuI* ends
KpnI
StuI
Cut with *KpnI* and *StuI*,
ligate in pMT- SUICIDE
1 restricted with *KpnI*
and *StuI*
SpeI
5' region
cat
pMT ΔthyA
V.cholerae
3360 bp
XhoI
KpnI
BglII
3' region
RP4 mob
R6K ori
BglII
StuI

FIG. 16

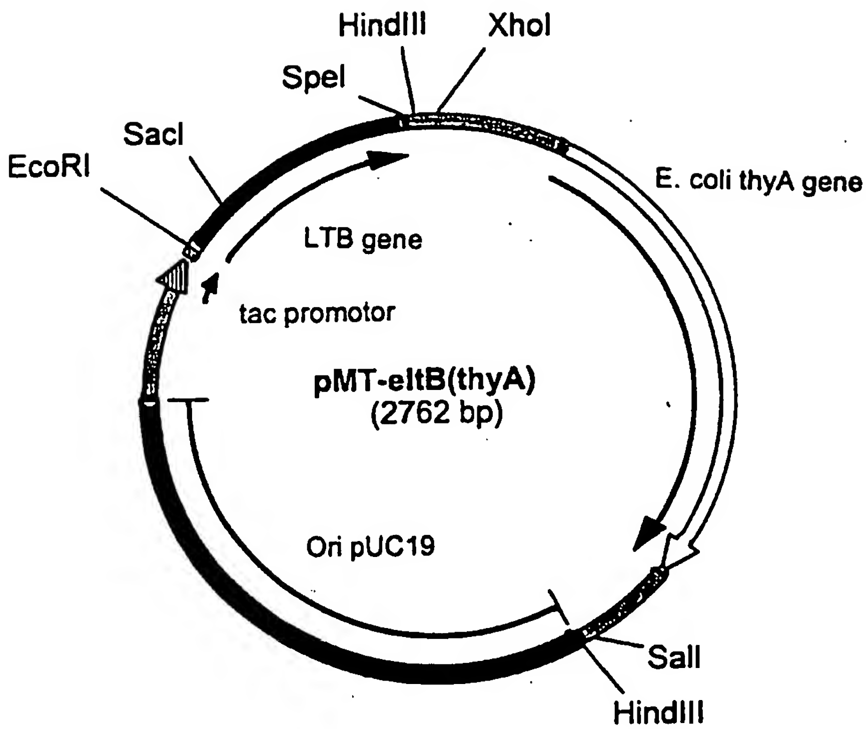
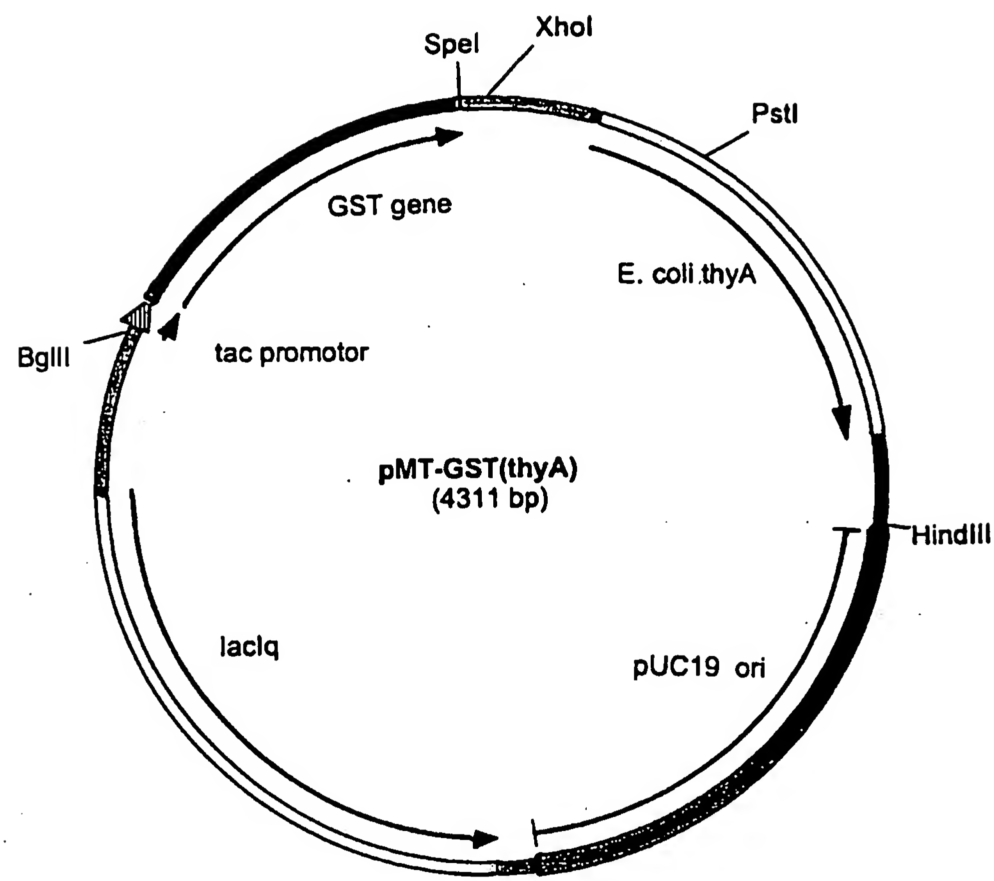

HindIII
XhoI
SpeI
SacI
EcoRI
E. coli thyA gene
LTB gene
tac promotor
pMT-eltB(thyA)
(2762 bp)
Ori pUC19
SalI
HindIII

FIG. 17


SpeI
XhoI
PstI
GST gene
E. coli thyA
BglII
tac promotor
pMT-GST(thyA)
(4311 bp)
HindIII
lacIq
pUC19 ori

Method of producing *thy* A⁻ strains of *Vibrio cholerae*, such strains and their use.

The present invention relates to a method of producing *thy* A $^-$ strains of *Vibrio cholerae*, such strains and their use. The invention particularly relates to a strain of *Vibrio cholerae* that has been deprived of its *thy* A gene in the chromosome, i.e. a Δ *thy* A strain lacking the functionality of the *thy* A gene. This strain may comprise one or several episomal autonomously replicating DNA elements, such as plasmids, having an optionally foreign, e.g. *E. coli*, functional *thy* A gene that enables the strain to grow in the absence of thymine in the growth medium, and optionally having a structural gene encoding a homologous or heterologous protein. The invention further relates to *thy* A nucleotide sequences and proteins encoded by them, and a vaccine comprising as an immunizing component a *Vibrio cholerae* Δ *thy* A strain of the invention or a *thy* A$^-$ strain of *V. cholerae* produced by the method of the invention.

Background.

The expression of recombinant genes in bacterial hosts is most often achieved by the introduction of episomal self-replicating elements (*e.g.* plasmids) that encode the structural gene of the protein of interest under the control of an appropriate promoter, into host bacteria. Such plasmids are most commonly maintained by the inclusion of selective marker genes that encode proteins that confer resistance to specific antibiotics (such as ampicillin, chloramphenicol, kanamycin, tetracycline etc.). They are then maintained in the host by addition of the appropriate antibiotic to the culture medium.

Stable maintenance of plasmids in host strains often requires the addition of the appropriate antibiotic selection without which they may segregate out giving rise to significant numbers of cells in any culture, that are devoid of plasmid and therefore cannot express the desired product.

However, the use of antibiotics in the production of recombinant proteins is undesirable for a number of reasons. Apart from the obvious increase in costs arising from the need to add them as a supplement to the growth medium, the use of antibiotics is considered a problem in the production of any recombinant protein intended for human or veterinary use. This is primarily for three reasons. Firstly, residual antibiotics can, in sensitive individuals, cause severe allergic reactions. Secondly, there is the possibility of selection for antibiotic resistant bacteria in the natural bacterial flora of those using the product, and finally, DNA encoding the antibiotic resistance may also be transferred to

sensitive bacteria in individuals using the product, thereby also spreading undesired antibiotic resistance in a cohort.

There are already inventions dealing with this problem, one such is the *par* gene which will effectively kill all cells that do not retain a copy of the plasmid after each cell division [1].

Another patent application [2], which touches on the invention described herein, was based on the knowledge of the *thy*A DNA sequence in *E. coli*. The authors introduced the *thy*A gene on a plasmid but used host strains that were spontaneous *thy*A$^-$ mutants selected on the bases of trimethoprim resistance. Such mutants are not well defined (carrying point mutations or small deletions) and may revert to the wild-type (*i.e. thy*A$^+$) at unacceptably high frequencies. This would lead to that the host bacteria could eliminate the plasmid and hence lose, or not give consistent and reliable, production of the desired recombinant product. An additional problem with trimethoprim selection is the possibility that resulting thymine dependence may arise due to a mutation in the dihydrofolate reductase (*fol*A) gene and hence not be complemented by a plasmid-borne *thy*A gene [3]. This patent application has been discontinued at least in Europe.

The use of *V.cholerae* for expression of recombinant genes has been shown to be advantageous over other prokaryotic expression systems in common use in that specific recombinant products may be produced in large quantities and secreted into the culture medium, thereby facilitating downstream purification procedures. This is in contrast to *E.coli* where the product often assembles in the periplasmic space [4]. One important factor endowing *V.cholerae* with this property is the *eps* genes in *V.cholerae* [5].

Thymidylate synthetase encoded by the *thy*A gene of *Escherichia coli* and other bacteria catalyses the methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP) and is an essential enzyme in the biosynthesis of deoxyribothymidine triphosphate (dTTP) for incorporation into DNA. In the absence of this enzyme the bacteria become dependent upon an external source of thymine which is incorporated into dTTP by a salvage pathway encoded by the *deo* genes [6].

Spontaneous mutants that are *thy*A$^-$ can be readily isolated on the basis of trimethoprim resistance. This antibiotic inhibits tetrahydrofolate regeneration from dihydrofolate produced by thymidylate synthetase-catalysed dTMP synthesis. Thus, if the cells are *thy*A$^-$ they become thymine dependent but no longer deplete the tetrahydrofolate pool in the presence of trimethoprim.

Description of the invention

The present invention is, in its different aspects, based on the novel nucleotide sequence of the *thy*A gene in *Vibrio cholerae*. A useful application of the *thy*A gene is e.g. in maintenance of recombinant plasmids employed in the overproduction of recombinant proteins in *V. cholerae*, and in the use of the sequence for insertion of foreign genes in a selectable and site-specific manner into the *V. cholerae* chromosome .

One aspect of the invention is directed to a method of producing a *thy* A^- strain of *Vibrio cholerae* comprising the step of site-directed mutagenesis in the *V. cholerae* chromosome for the deletion and/or insertion of gene nucleotides at the locus of the *thy* A gene having essentially the nucleotide sequence SEQ ID NO: 1 of FIG. 1.

The expression "having essentially the nucleotide sequence" in this specification and claims is intended to comprise nucleotide sequences which have some natural or unnatural nucleotide extensions, truncations, deletions or additions that do not interfere with the natural function of the nucleotide sequence in question.

Another aspect of the invention is directed to a *Vibrio cholerae thy* A^- strain which is a Δ *thy* A strain lacking the functionality of the *thy* A gene.

In an embodiment of this aspect of the invention the Δ *thy* A strain of *V. cholerae* comprises one or several episomal autonomously replicating DNA elements having a functional *thy* A gene that enables the strain to grow in the absence of thymine in the growth medium.

In a preferred embodiment the episomal autonomously replicating DNA element is a plasmid.

In another preferred embodiment the Δ *thy* A strain according to the invention comprises in an episomal autonomously replicating DNA element, especially a plasmid, a foreign *thy* A gene, such as an *E. coli* gene.

In a particularly preferred embodiment of this aspect of the invention the Δ *thy* A strain according to the invention comprises in one or several episomal autonomously replicating DNA elements, especially plasmids, in addition to a foreign *thy* A gene, such as an *E. coli* gene, also a structural gene encoding a homologous or heterologous protein, such as heat labile enterotoxin B-subunit of *Escherichia coli* (LTB) or *Schistosoma japonicum* glutathione S-transferase 26 kD protein (GST 26 kD).

A third aspect of the invention is directed to a nucleotide sequence of a 5'-flanking region of a structural *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 2 of FIG. 2.

A fourth aspect of the invention is directed to a nucleotide sequence of a 3'-flanking region of a structural *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 3 of FIG. 3.

The nucleotide sequence SEQ ID NO: 1, is useful for insertion of foreign genes in a selectable and site-specific manner into the *V. cholerae* chromosome, and for site-directed mutagenesis in the production of *Vibrio cholerae thy* A^- strains.

A fifth aspect of the invention is directed to a protein encoded by a nucleotide sequence of a *thy* A gene of *Vibrio cholerae* according to the invention, such as a protein having the amino-acid sequence SEQ ID NO: 4 of FIG. 4.

A sixth aspect of the invention is directed to a protein encoded by a nucleotide sequence of a 5'- flanking region of a structural *thy* A gene of *Vibrio cholerae* according to the invention, such as the protein having the amino-acid sequence SEQ ID NO: 5 of FIG. 5.

The proteins according to the fifth and sixth aspect of the invention are each useful for research purposes, and potential targets for anti-microbial therapy.

A seventh aspect of the invention is directed to a vaccine comprising as an immunising component a *Vibrio cholerae* Δ *thy* A strain according to the invention or a *thy* A^- strain of *Vibrio cholerae* produced by the method of the invention. The vaccine will be used for prophylactic and therapeutic treatment of cholera and optionally other infectious diseases, especially in cases where the used strain has been engineered to express foreign proteins. The vaccine will in addition to the immunising component(s) comprise a vehicle, such as physiological saline solution, and other components frequently used in vaccines such as buffers and adjuvants. Useful vehicles, buffers, adjuvants and other components are disclosed in e.g. the European and US Pharmacopoeia.

Short description of the drawings

Figure 1 shows the nucleotide sequence SEQ ID NO:1 of the *thy* A gene of *Vibrio cholerae.*

Figure 2 shows the nucleotide sequence SEQ ID NO:2 of the 5'-flanking region of the structural *thy* A gene of *Vibrio cholerae.*

Figure 3 shows the nucleotide sequence SEQ ID NO:3 of the 3'-flanking region of the structural *thy* A gene of *Vibrio cholerae.*

Figure 4 shows the amino-acid sequence SEQ ID NO:4 of the protein encoded by the structural *thy* A gene of *Vibrio cholerae* .

Figure 5 shows the amino-acid sequence SEQ ID NO:5 of the protein encoded by the 5'-flanking region of the structural *thy* A gene of *Vibrio cholerae*.

Figure 6 shows the cloning of a *Eco*RI/*Hind*III fragment containing the *V.cholerae thy*A gene in pUC19.

Figure 7 shows a comparison of *thy*A gene products from *E. coli* [16], *V. cholerae* and *H. influenzae* [17] showing the high degree of homology between *V. cholerae* and *H. influenzae* compared with *E. coli*.

Figure 8 shows the insertion of a Kan^{R}-resistance gene block in the *Pst*I site of the *V.cholerae thy*A gene in pUC19.

Figure 9 shows PCR to generate a *thy*A -Kan fragment with *Xba*I ends.

Figure 10 shows ligation of the *thy*A-Kan fragment with *Xba*I ends in plasmid pNQ705.

Figure 11 shows partial deletion of the *thy*A gene and the start of the Kan gene in pNEB193.

Figure 12 shows *Xba*I cleavage to excise the Δ*thy*A Δkan gene from pNEB193, ligation into *Xba*I restricted pDM4.

Figure 13 shows an outline of a strategy to completely delete the *thy*A gene of *V. cholerae.*

Figure 14 shows insertion of the 5' region upstream of *thy*A in pMT-SUICIDE 1; generation of pMT with 5 prim.

Figure 15 shows insertion of the 3' region downstream of *thy*A in pMT with 5 prim; generation of pMT Δ*thy*A *V.cholerae.*

Figure 16 shows the expression vector pMT-eltB(thyA) used for expression of LTB in *V. cholerae* JS1569 Δ*thyA*.

Figure 17 shows the expression vector pMT-GST(thyA) used for expression of GST in *V. cholerae* JS1569 Δ*thy*A.

<u>Description of experiments</u>

Strategy employed

In order to produce defined *thy*A mutants of *V. cholerae* that could be used as suitable production strains for recombinant proteins encoded on plasmids maintained by *thy*A complementation, it was first necessary to clone and characterise the wild-type gene and its

5' and 3' flanking regions. Our strategy was to first clone the *thy*A gene of *V. cholerae* on a plasmid, on the basis of complementation of the *thy*A auxotrophy in a strain of *E.coli* K12. Restriction analysis and subcloning experiments were done in order to locate the *thy*A structural gene on the large DNA fragment initially obtained. The appropriate region containing the *thy*A gene and it's 5' and 3' flanking regions gene was then sequenced.

To verify that one of the sequenced genes was in fact the *thy*A gene of *V. cholerae*, homology comparisons were made with *thy*A sequences from other organisms. The cloned gene could also complement the *thy*A phenotype of a *V. cholerae* mutant strain that had been selected on the basis of trimethoprim resistance. Sequence analysis of this mutant showed that it did indeed have a single base change in the gene we had identified as *thy*A, which resulted in a stop codon giving a non-functional truncated gene product.

Knowledge of the *thy*A sequence and that of the region surrounding it allowed the use of suitable suicide vectors for site-directed mutagenesis. Strategies considered were (a) insertional inactivation (b) a combination of insertional inactivation and gene deletion and (c) removal of the entire gene:

(a) Insertional inactivation of the *thy*A gene was achieved by insertion of a Kan^R gene block (with the suicide vector pNQ705 [14].

(b) A deletion of approximately 400 bp was made in the strain carrying the Kan^R geneblock that removed 200 bp each from the *thy*A gene upstream of the insertion site and from the kanamycin resistance gene which was thereby inactivated. We thus obtained a deleted *thy*A gene where the deletion was in the central part of the gene and followed by an insertion of a non-coding region of DNA. This construct was inserted into the *V .cholerae* chromosome using the suicide vector pDM4 and resulted in a strain called JS1569 Δ*thy*AΔKan.

(c) Complete removal of the *thy*A gene was done by ligating together the regions flanking the structural gene, taking care not to disrupt other open reading-frames (disruption of the adjacent *lgt* gene is also lethal). The DNA carrying the deletion was cloned into a novel suicide vector (PMT-SUICIDE-1) used for insertion of the sequence into the *V. cholerae* chromosome. The resulting strain is called JS1569 Δ*thy*A.

For expression of recombinant genes in these Δ*thy*A strains of *V. cholerae*, two expression vectors were constructed. Each consisted of the *thy*A gene from *E. coli*, the origin of replication of the general purpose high copy-number vector pUC19, the *tac* promotor and the rho-independent *trp*A transcription terminator. In one of the two vectors the *lac*I^q gene

had been inserted in order to regulate expression from the *tac* promotor which also contained the *lac* operator sequence.

Two genes were cloned into these plasmids and expressed in the newly generated *thy*A -deleted strain of *V. cholerae*; JS1569Δ*thy*A. The first encoded the B subunit of human heat-labile enterotoxin from *E. coli* (LTB) (Figure 16), the second was the sj26 glutathione-S-transferase (GST) from *Schistosoma japonicum* (Figure 17).

LTB is similar in structure to the B subunit of cholera toxin naturally produced by the host strain and was secreted into the growth medium. The other protein is eukaryotic in origin, coming from the Asian Liver Fluke. Sj26 GST is known to express to high levels in *E. coli* and accumulates in the cytoplasm. Expression of the two recombinant proteins was assessed on the basis of GM1 ELISA of the culture supernatant in the case of LTB and a commercially available assay in the case of GST. Both proteins were also analysed on the basis of SDS-PAGE and Western blots.

Origin of the *thy*A gene

The *thy*A gene was cloned from strain *V.cholerae* JS1569. This strain originates from the *V.cholerae* Inaba strain 569B of the classical biotype (ATCC No 25870). The strain has a deletion in the *ctx*A gene [7] and has been made rifampicin resistant [8].

Cloning of a 1.4 kB *Hind*III/*Eco*RI fragment encompassing the *V.cholerae thy*A gene.

Chromosomal DNA prepared by the CTAB method [9] was digested to completion with the restricion enzyme *Hind*III.

The digested DNA was ligated into the general purpose vector plasmid pBR322 (New England Biolabs Inc. Beverly, MA USA) which had been digested with *Hind*III and treated with alkaline phosphatase.

The ligation mixture was electroporated [10] into a *E.coli* HB101 strain that was phenotypically ThyA$^-$ (selected on the basis of trimethoprim resistance) and the culture spread onto modifed Syncase (MS) agar plates [11] supplemented with 50 μg/ml ampicillin, but containing no thymine. Thus transformants were selected both on the basis of plasmid acquisition and the presence of a functional *thy*A gene.

Colonies that grew up were streaked out to single colonies on the same type of agar plates, and then grown up in MS broth supplemented with ampicillin. Plasmid DNA was prepared by "Wizard miniprepps" (ProMega Corp. Madison Wis.) and digested with *Hind*III. A fragment of approx. 10-12 kB was isolated, this clone was namned ThyA B2.

To reduce the size of the fragment, the plasmid was cut with *Eco*RI and religated using T4 ligase. The ligated DNA was again electroporated into the *E.coli* strain described above using the same selective conditions for growth of transformants.

Colonies resulting from this experiment were isolated as described above and plasmid DNA purified and anlysed by double digest with *Eco*RI and *Hind*III. A DNA fragment of approximately 1.4 kb remained which retained the ability to complement the *thy*A mutation in the *E. coli* host strain. This fragment was cloned into the plasmid pUC19 (New England Biolabs) that had been digested with the same two enzymes and treated with alkaline phosphatase. Following electroporation, transformants from the experiment were isolated and characterised as described above. This clone was called ThyA 1:2 (Figure 6).

Verification that the 1.4 kB *Hind*III/*Eco*RI fragment contains the *thy*A gene.

Southern blot analysis. To verify that the cloned fragment was indeed from *V.cholerae* chromosomal origin, DNA from strain JS1569 was digested to completion with *Hind*III and *Eco*RI and *Hind*III. The DNA fragements were resolved by agarose electrophoresis together with *Hind*III digested clone ThyA B2 and *Eco*RI and *Hind*III digested clone ThyA 1:2.

After electrophoresis the DNA was transferred to a Nylonmembrane, immobilised by UV irradiation and hybridised (under stringent conditions) with the 1.5 kB fragment excised from clone ThyA 1:2 that had been labelled with ^{32}P dCTP using Amershams Multiprime kit.

Results. In both *Hind*III digested chromosomal DNA and in *Hind*III digested clone ThyA B2 an approx. 10 kB band was evident. Likewise in *Eco*RI/*Hind*III digested chromosomal DNA and clone ThyA 1:2 plasmid DNA a 1.4 kB band was evident (data not shown). These data demonstrated that the cloned fragment was derived from *V.cholerae* JS1569 DNA.

Transformation of JS1569 ThyA$^-$ with the plasmid ThyA 1:2.

To verify that the 1.4 B cloned *Eco*RI/*Hind*III fragment could support growth of phentotypically ThyA$^-$ *V.cholerae*, a thymine dependent mutant of JS1569 (*V.cholerae* JS1569 4.4) was electroporated with the plasmid ThyA 1:2. Electroporation and selective media were as described above. JS1569 4.4 does not grow on MS medium without the addition of thymine.

Results. Colonies of JS1569 4.4 were isolated that grew in the absence of thymine. All were shown to harbour the ThyA 1:2 plasmid, thus supporting the assumption that the cloned fragment contained the *thy*A gene from *V.cholerae*.

DNA sequencing of the plasmid ThyA 1:2. Plasmid DNA was sequenced by the dideoxy chain termination method [12] using the ABI PRISM™ Dye terminator cycle sequencing kit (Perkin Elmer). Both commercially available as well as custom made primers were used. The DNA sequences were analysed on an ABI PRISM 373 automatic sequencer (Perkin Elmer). Data were analysed using the AutoAssembler Software package (Perkin Elmer). Homology searches with the found DNA sequence were done with the GCG program [13].

Results. The best homologies were with thymidylate synthetases from various species. Note that the homology with *E.coli* thymidylate synthetase is rather weak. (Figure 7)

Strategy for deletion of the *thy*A gene in *V.cholerae* JS1569.

Two different strategies were used for obtaining defined *thy*A mutants of *V. cholerae* JS1569, the first involved inactivation of the *thy*A gene by insertion of a Kan^R gene block followed by partial deletion of the *thy*A gene and the Kan^R gene block.

The second strategy was directed to completely delete the *thy*A gene from the chromosome by means of a novel suicide vector pMT SUICIDE-1. This vector contains the 5' and 3' flanking regions of the *thy*A gene as well as the R6K origin of replication and the RP4 *mob* genes.

To replace the *thy*A gene of strain JS1569 we decided to use the already thymine-dependent JS1569 4.4 since preliminary experiments indicated that there is a strong selective disadvantage to go from wildtype to thymine dependence even in the presence of high levels of exogeneous thymine.

Inactivation of the *thy*A gene by insertion of a Kan^R gene block

Our strategy involved inactivation of the *thy*A gene by insertion of a kanamycin resistance gene into a unique *Pst*I site in the *thy*A gene in the form of a Kan^R gene block (Pharmacia) (Figure 8). This construct was amplified by PCR (Expand™High Fidelity PCR system Boehringer Mannheim) with primers that incorporate *Xba*I ends so that it could be transferred into the suicide plasmid pNQ705 [14] which carries a unique *Xba*I site and the chloramphenicol resistance gene.

The following primers were used for PCR amplification of the insertionally inactiviated gene:

ThyA-10: $^{5'}$GCT **CTA GAG** CCT TAG AAG GCG TGG TTC$^{3'}$
corresponding to bases 557 to 575 in SEQ ID NO: 2 (Figure 2) with an added *Xba*I site (in bold)
and
ThyA-11: $^{5'}$GCT **CTA GAG** CTA CGG TCT TGA TTT ACG GTA T$^{3'}$
corresponding to the complementary sequence of bases 235 to 257 in SEQ ID NO:2 (Figure 3) with an added *Xba*I site (in bold) (Figure 9 + 10).

The resulting plasmid was then transferred to the *E.coli* S-17 that was used in conjugation experiments.

Since the recipient strain JS1569 4.4 is rifampicin resistant and chloramphenicol sensitve and the donor strain *E.coli* S-17 is both chloramphenicol and kanamycin resistant, transconjugants were selected by selection for resistance to both rifampicin and kanamycin.

The resulting *V. cholerae* strains however would also be chloramphenicol resistant since the entire plasmid would initially be inserted into the chromosome.

Exconjugants that had incorporated the inactivated *thy*A gene carrying the Kan^R geneblock into the chromosome and lost the pNQ705 plasmid could then be selected among those that were chloramphenicol sensitive but remained kanamycin resistant.

To verify insertion of the Kanamycin reiststance gene in the *thy*A gene the entire *thy*A gene was PCR amplified with primers thyA-10 and thyA-11, and the size of the resulting fragment compared to that of the native *thy*A gene . The expected *thy*A fragment of 2.6 kb compared to that of the native *thy*A gene of 1.4 kb was found.

Results. Exconjugants were shown to be kanamycin resistant, chloramphenicol sensitive and when amplified by PCR, shown to have incorporated the kanamycin resistance gene block into the chromosome. Sequencing of the amplified fragment showed that the only defect in the gene was due to the insertion of the kanamycin gene. This indicated that the recombination event that had incorporated the insertionally inactivated gene into the chromosome had also eliminated the point mutation that had made the recipient strain (JS1569 4.4) thymine dependent. Growth of the resulting strain was only observed if the growth medium was supplemented with thymine (200 µg/ml).

Partial deletion of the *thy*A gene and the Kan^R gene block

To further ensure a nonreversible *thy*A mutation the insertionally inactivated *thy*A was subcloned as a *Xba*I fragment into pNEB 193 (New England Biolabs). PCR

primers were designed that deleted 209 basepairs from the *thy*A gene and removed 261 basepairs from the Kan^R geneblock.

Thus the *thy*A gene was further disrupted and that the kanamycin resistance gene was also inactivated (by removal of the start of the coding region). The overall result of this procedure was a strain carrying a deleted *thy*A gene that also contained an insertion of noncoding DNA.

ThyA-14: $^{5'}$GGG GGC **TCG AGG** GGC ACA TCA CAT GAA$^{3'}$
ThyA-15: $^{5'}$CCC CCC **TCG AGC** GCC AGA GTT GTT TCT GAA$^{3'}$

Letters in **bold** indicate *Xho*I cleavage sites (Figure 11).

After PCR amplification a DNA fragment was obtained encompassing the entire plasmid with exception of the deleted region. The amplified DNA was digested with *Xho*I, self ligated and transformed into *E.coli* HB101. Colonies were selected for on plates containing ampicillin. Individual colonies were selected and restreaked. Small-scale plasmid preparations from individual colonies yielded the expected restriction patterns when anlysed with *Xba*I, *Xho*I, *Hind*III and *Rsa*I restriction enzymes.

The incomplete *thy*A gene carrying an inactivated kanamycin resistance gene was cut out from the vector by *Xba*I digestion, purified and ligated into pDM4 [15] (Figure 12). PDM4 is a suicide vector derived from pNQ705 containing the *Sac*BR gene from *Bacillus subtilis* and a modified multicloning site.

After transfer of the pDM4 (Δ*thy*AΔKan) plasmid to the *E.coli* S-17 strain a transconjugation experiment was performed. This time the *V.cholerae* JS1569 *thy*AKan strain obtained above was used as recipient strain.

The mating was done as described above with selection for rifampicin and chloramphenicol. After growth in this medium colonies were selected on medium containing 10% sucrose in the absence of chloramphenicol. Sucrose induces the *sac*BR gene which encodes levansucrase that converts sucrose to levan. This compound is toxic to many Gram negative organisms. In this way clones still carrying the suicide plasmid were killed leaving exconjugants that had lost the plasmid.

Results. A colony was selected that was chloramphenicol and kanamycin sensitive. PCR amplification of the *thy*A region with the primers ThyA-10 and thyA-11

confirmed that the *thy*AKan fragment (2.6 kb) on the chromosome had been replaced with the Δ*thy*AΔKan fragment (2.1 kb).

Growth of the resulting strain was only observed if the growth medium was supplemented with thymine (200 µg/ml). This strain was named *V.cholerae* JS1569 Δ*thy*AΔKan.

Direct deletion of the *thy*A gene in *V. cholerae*.

For this approach the 5' and 3' sequences flanking the *thy*A gene were used. A novel suicide vector was constructed, pMT SUICIDE-1 (Fig 14) that contains the R6K origin of replication, the *mob* genes from RP4, a chloramphenicol resistance gene and a multicloning site from Litmus 28 (New England Biolabs). Effectively, a modified fragment was constructed in which the *thy*A coding region was replaced by a multicloning site (derived from Litmus 28) leaving only the 5' and 3' region of the *thy*A locus from *V.cholerae*. The resulting plasmid was used to generate a *V. cholerae* strain in which the entire *thy*A gene had been deleted.

As starting material for this construction the pMT SUICIDE-1 plasmid was used (M. Lebens, unpublished).

From the 5' and 3' regions of the *thy*A locus the following PCR primers were designed:

ThyA-33: 5'GGA **CTA GTG** GGT TTC CTT TTT GCT AT3'

corresponding to bases 109 to 126 in the SEQ ID NO:2 (figure 2) (5' region of the *thy*A region) with a *Spe*I site (indicated in bold) and

ThyA-34: 5'CCC CGC **TCG AGA** CCC TAT TTT GCT GCT AC3'
corresponding to the complementary sequence of base 815 to 832 in the SEQ ID NO:2 with a *Xho*I site (indicated in bold) attached to it.

This primer pair gives a PCR fragment of 743 bases corresponding to the 5' flanking region of the *thy*A gene.

ThyA-31: 5'CGG **GGT ACC** TGG CTT GAT GGG TTT TAT3'

corresponding to bases 22 to 39 in the SEQ ID NO:3 (figure 3) (3' region of the *thy*A region) with a *Kpn*I site (indicated in bold) and

ThyA-32: 5'GAA **GGC CTT** CGC CTC TGC TTG CGA CT3'

corresponding to the complementary sequence of bases 731 to 749 in the SEQ ID NO:3 with a *Stu*I site (indicated in bold).
This primer pair gives a PCR fragment of 746 bases corresponding to the 3' flanking region of the *thy*A gene.

As template for the PCR reactions a chromosomal DNA preparation from *V. cholerae* JS1569 was used (Figure 13).

The amplified DNA were digested with the appropriate restriction enzymes and cloned into the pMT-SUICIDE 1 vector (Figure 14 and 15) yielding the plasmid pMTΔ*thy*A *V.cholerae* that contains approximately 700 base-pairs of the 5' region upstreams of the *thy*A gene and the same number of base-pairs of the 3' region downstreams of the *thy*A gene.

This plasmid was transferred to *E.coli* S17-1 and used in conjugation experiments as described above. As recipient the *V. cholerae* JS1569 4.4 strain was used. Matings were done on LB agar supplemented with rifampicin, chloramphenicol and thymine. Exconjugants that had lost the suicide plasmid from the chromosome were selected on the basis of chloramphenicol sensitivity.

Results. A chloramphenicol sensitive and rifampicin resistant colony was selected. PCR amplification with the primers ThyA-10 and ThyA-11 of the *thy*A region resulted in a 1.4 kb fragment from the native *thy*A gene and a 0.6 kb fragment from the Δ*thy*A gene.This confirmed that the *thy*A structural gene on the chromosome had been deleted. Furthermore the bacteria could only grow in medium complemented with thymine. This strain is namned *V. cholerae* JS1569 Δ*thy*A.

Expression of the B subunit of heat-labile enterotoxin from *E. coli* (LTB) and the sj26 glutathione-S-transferase (GST) from *Schistosoma japonicum* in *V. cholerae JS1569* Δ*thy*A..

Two expression vectors were constructed, each consisted of the *thy*A gene from *E. coli*, the origin of replication of the high copy-number vector pUC19, the *tac* promotor and the rho-independent *trp*A transcription terminator. In one of the two vectors the *lac*I^q gene had been inserted in order to regulate expression from the *tac* promotor which also contained the *lac* operator sequence (figure 16 and 17).

Expression of the LTB protein in *V.cholerae* JS1569 Δ*thy*A strain.

The expression vector shown in figure 16 was electroporated into *V.cholerae* JS1569 Δ*thy*A. Transformants were selected for on MS -agar. Individual colonies were grown up to produce mini-plasmid preps that were checked by restriction enzyme analysis. For expression a transformant was grown in MS medium at 37°C in a shaker culture. The culture medium was harvested and assayed for LTB by the GM1- ELISA.

Results. The culture was found to produce approximately 300 μg/ml of LTB as assayed by the GM1 ELISA. SDS-PAGE and Western blot using an LTB specific monoclonal antibody further verified that the secreted protein was LTB.

Expression of the GST protein in *V.cholerae* JS1569 Δ*thy*A strain

The sj26 glutathione-S-transferase (GST) from *Schistosoma japonicum* was cloned in the expression vector shown in figure 17. This vector is identical to the first except for the sequence of the *lac*I^q gene. The *lac*I^q allows for controlled expression of recombinant proteins. The vector was electroporated into *V.cholerae* JS1569 Δ*thy*A . Transformants were selected for on MS -agar. Indivudual colonies were grown up to produce mini-plasmid preps that were checked by restriction enzyme analysis. For expression a transformant was grown in MS medium at 37°C in a shaker culture with addition of IPTG.

Results. The recombinant protein was found in the cytoplasm of the *V. cholerae* bacteria. SDS-PAGE and Western blot with a GST specific monoclonal antibody (Pharmacia BioTech, Uppsala) confirmed that GST was expressed. The level of GST expression was more difficult to determine than for LTB since the protein was expressed intracellulary but was judged to be in the same range as for LTB.

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CLAIMS

1. A method of producing a *thy* A⁻ strain of *Vibrio cholerae* comprising the step of site-directed mutagenesis in the *V. cholerae* chromosome for the deletion and/or insertion of gene nucleotides at the locus of the *thy* A gene having essentially the nucleotide sequence SEQ ID NO: 1 of FIG. 1.

2. A *Vibrio cholerae thy* A⁻ strain which is a Δ *thy* A strain lacking the functionality of the *thy* A gene.

3. A Δ *thy* A strain of *Vibrio cholerae* according to claim 2 comprising one or several episomal autonomously replicating DNA elements having a functional *thy* A gene that enables the strain to grow in the absence of thymine in the growth medium.

4. A Δ *thy* A strain of *Vibrio cholerae* according to claim 3, wherein the episomal autonomously replicating DNA element is a plasmid.

5. A Δ *thy* A strain of *Vibrio cholerae* according to claim 3 or 4 comprising a foreign *thy* A gene.

6. A Δ *thy* A strain of *Vibrio cholerae* according to claim 5, wherein the foreign *thy* A gene is an *E. coli* gene.

7. A Δ *thy* A strain of *Vibrio cholerae* according to any one of claims 3 to 6, wherein the one or several episomal autonomously replicating DNA elements also comprise a structural gene encoding a homologous or heterologous protein.

8. A Δ *thy* A strain of *Vibrio cholerae* according to claim 7, wherein the encoded protein is selected from heat labile enterotoxin B-subunit of *Escherichia coli* (LTB) and *Schistosoma japonicum* glutathione S-transferase 26 kD protein (GST 26 kD).

9. A nucleotide sequence of a *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 1 of FIG. 1.

10. A nucleotide sequence of a 5'- flanking region of a structural *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 2 of FIG. 2.

11. A nucleotide sequence of a 3'- flanking region of a structural *thy* A gene of *Vibrio cholerae* having essentially the nucleotide sequence SEQ ID NO: 3 of FIG. 3.

12. A protein encoded by a nucleotide sequence of a *thy* A gene of *Vibrio cholerae* according to claim 9.

13. A protein according to claim 12, wherein the protein has the amino-acid sequence SEQ ID NO: 4 of FIG. 4.

14. A protein encoded by a nucleotide sequence of a 5'- flanking region of a structural *thy* A gene of *Vibrio cholerae* according to claim 10.

15. A protein according to claim 14, wherein the protein has the amino-acid sequence SEQ ID NO: 5 of FIG. 5.

16. A vaccine comprising as an immunising component a *Vibrio cholerae* Δ *thy* A strain according to any one of the claims 2 - 8 or a *thy* A^- strain of *Vibrio cholerae* produced by the method of claim 1.

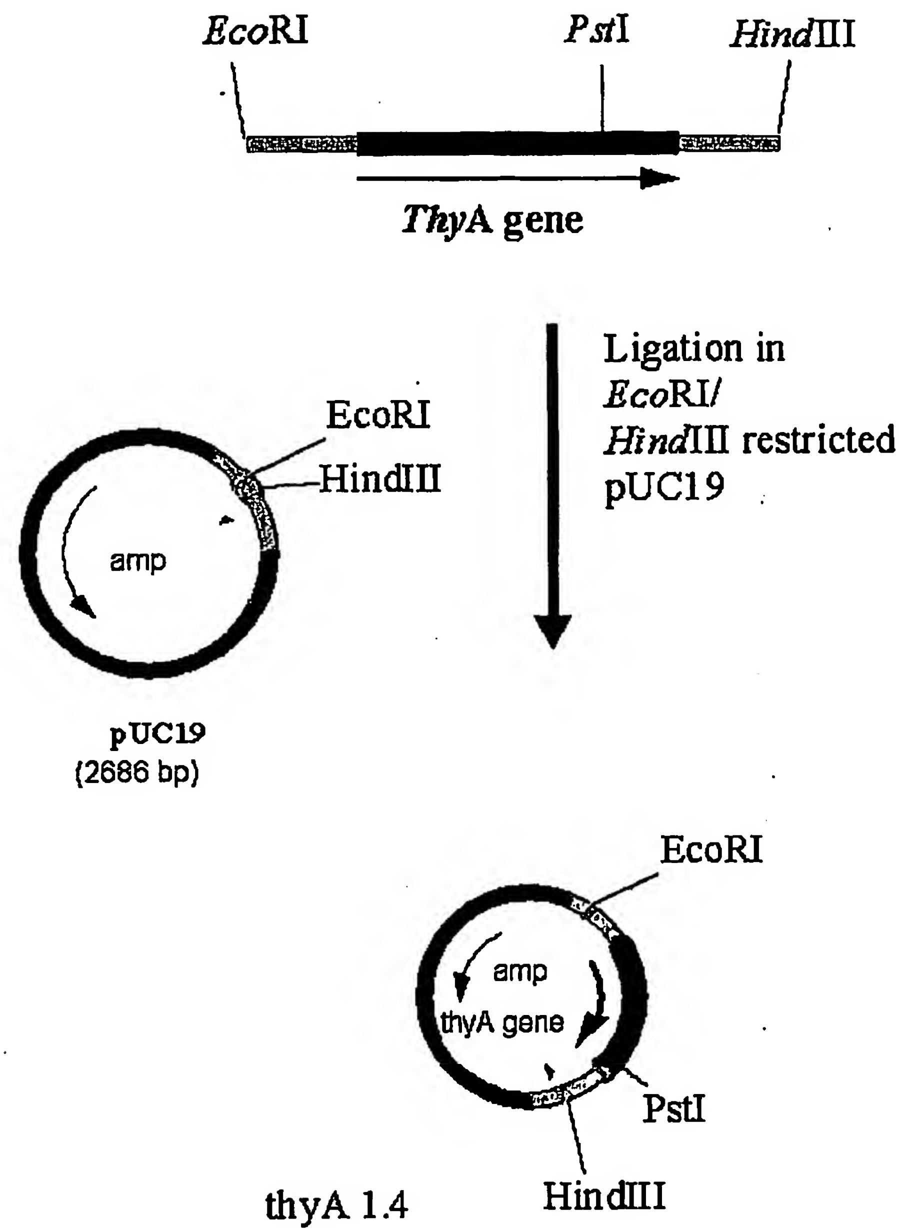

EcoRI
PstI
HindIII
ThyA gene
Ligation in
EcoRI/
HindIII restricted
pUC19
EcoRI
HindIII
amp
pUC19
(2686 bp)
EcoRI
amp
thyA gene
PstI
HindIII
thyA 1.4

Fig. 1

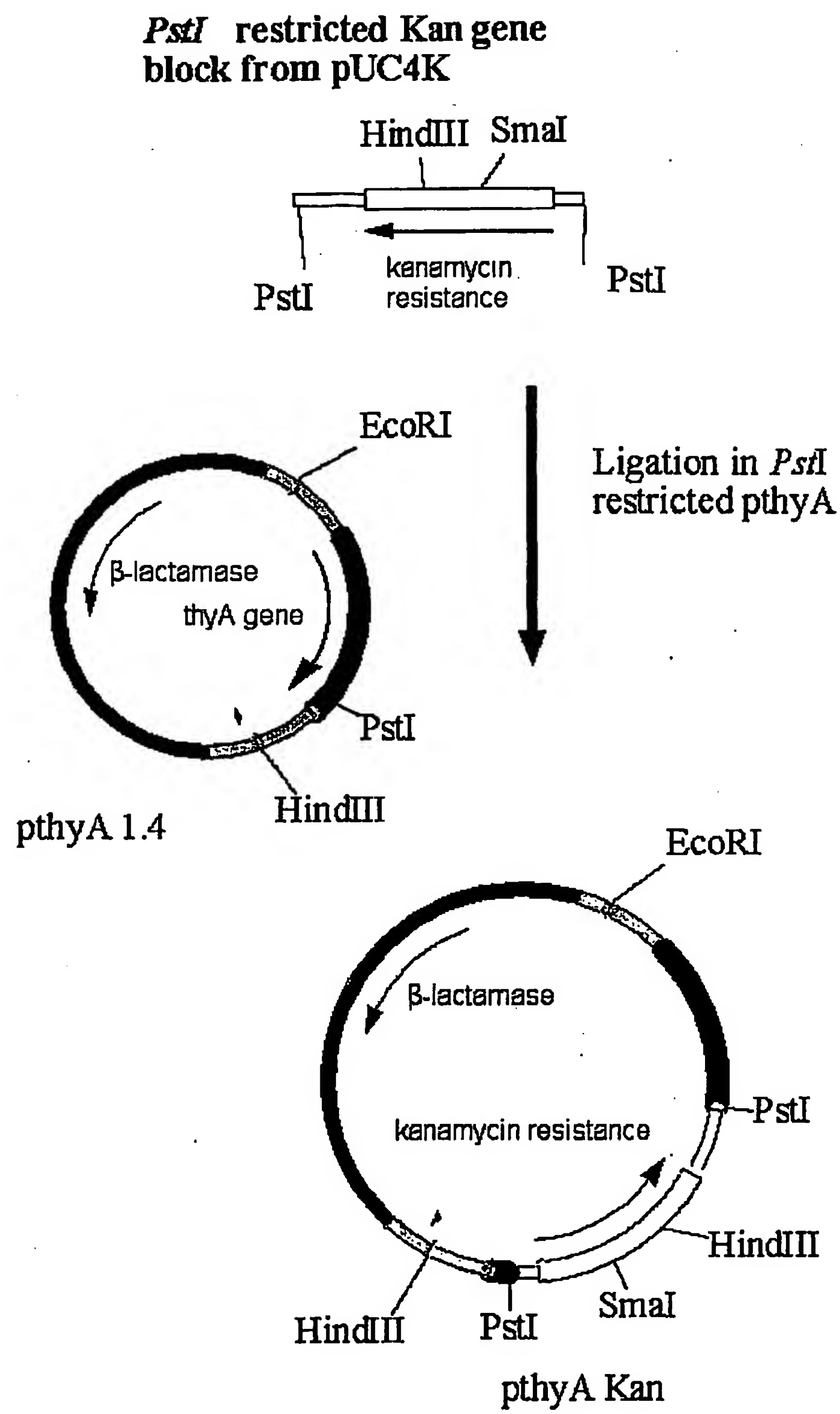
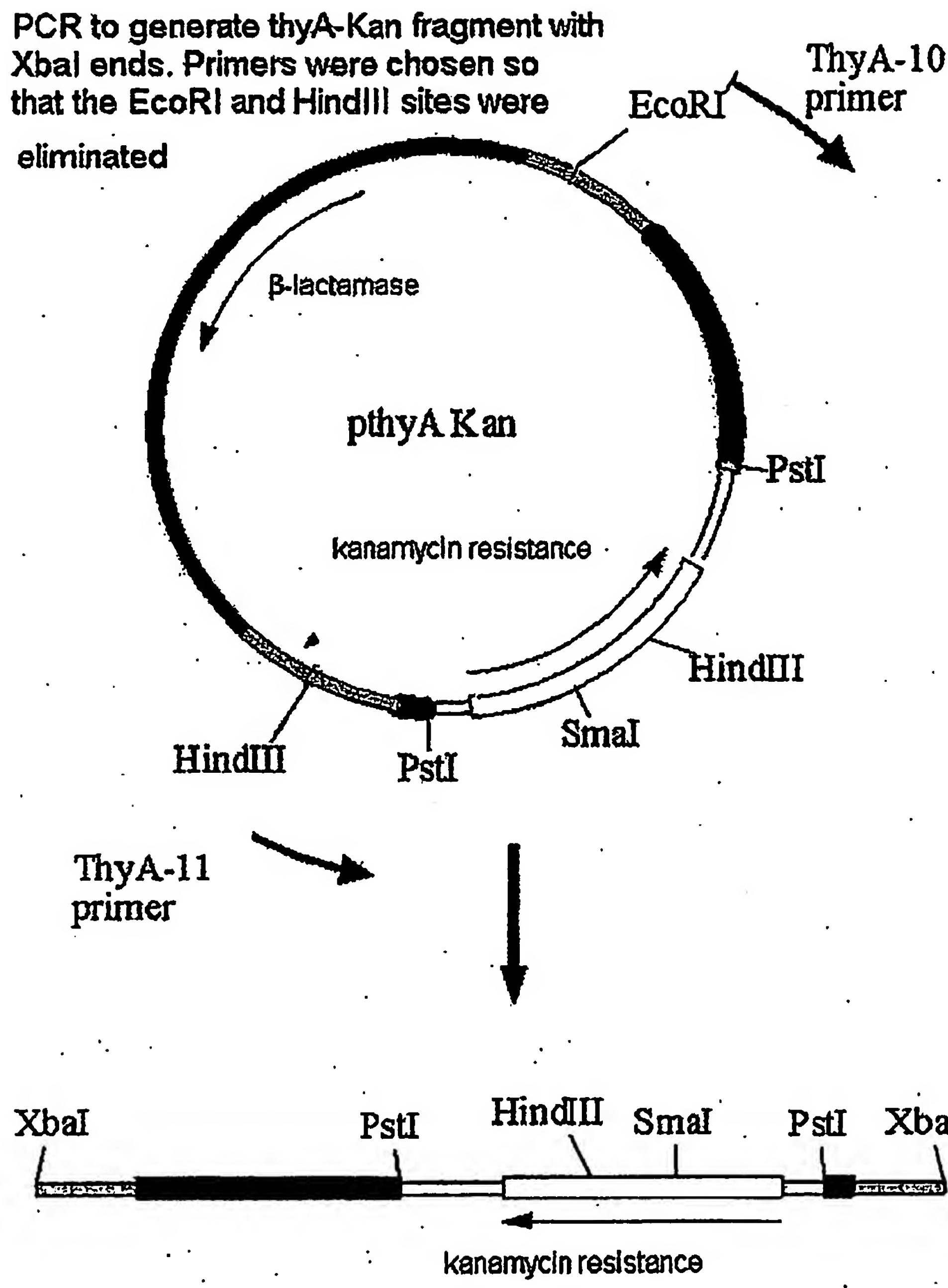

PstI restricted Kan gene block from pUC4K
HindIII SmaI
PstI
kanamycin resistance
PstI
Ligation in PstI restricted pthyA
EcoRI
β-lactamase
thyA gene
PstI
HindIII
pthyA 1.4
EcoRI
β-lactamase
kanamycin resistance
PstI
HindIII
SmaI
PstI
HindIII
pthyA Kan

Fig. 2



thyA Kan XbaI ends

Fig. 3

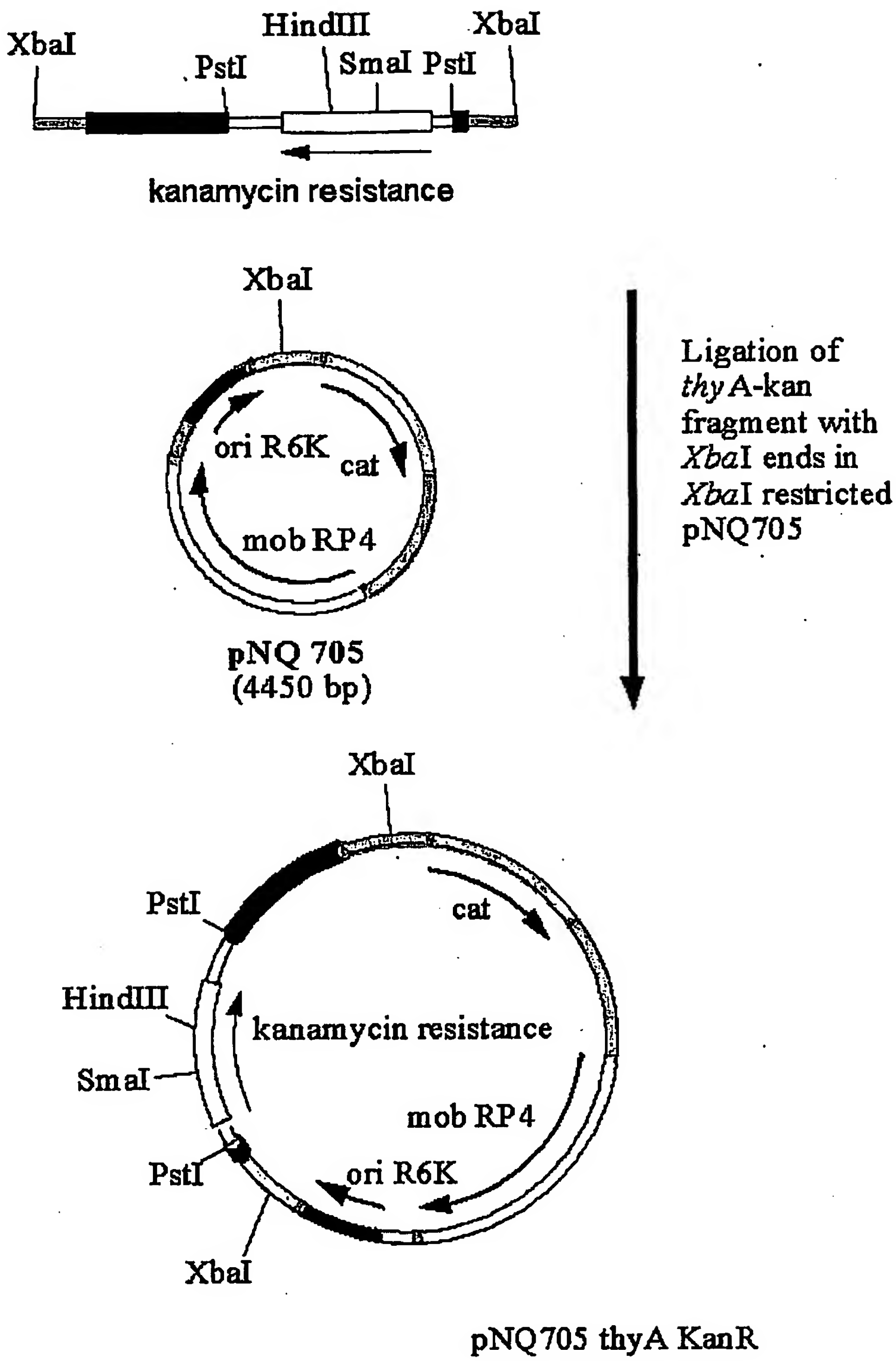

XbaI
HindIII
XbaI
PstI
SmaI PstI
kanamycin resistance
XbaI
ori R6K
cat
mob RP4
pNQ 705
(4450 bp)
Ligation of
*thy*A-kan
fragment with
*Xba*I ends in
*Xba*I restricted
pNQ705
XbaI
PstI
cat
HindIII
kanamycin resistance
SmaI
mob RP4
PstI
ori R6K
XbaI
pNQ705 thyA KanR

Fig. 4

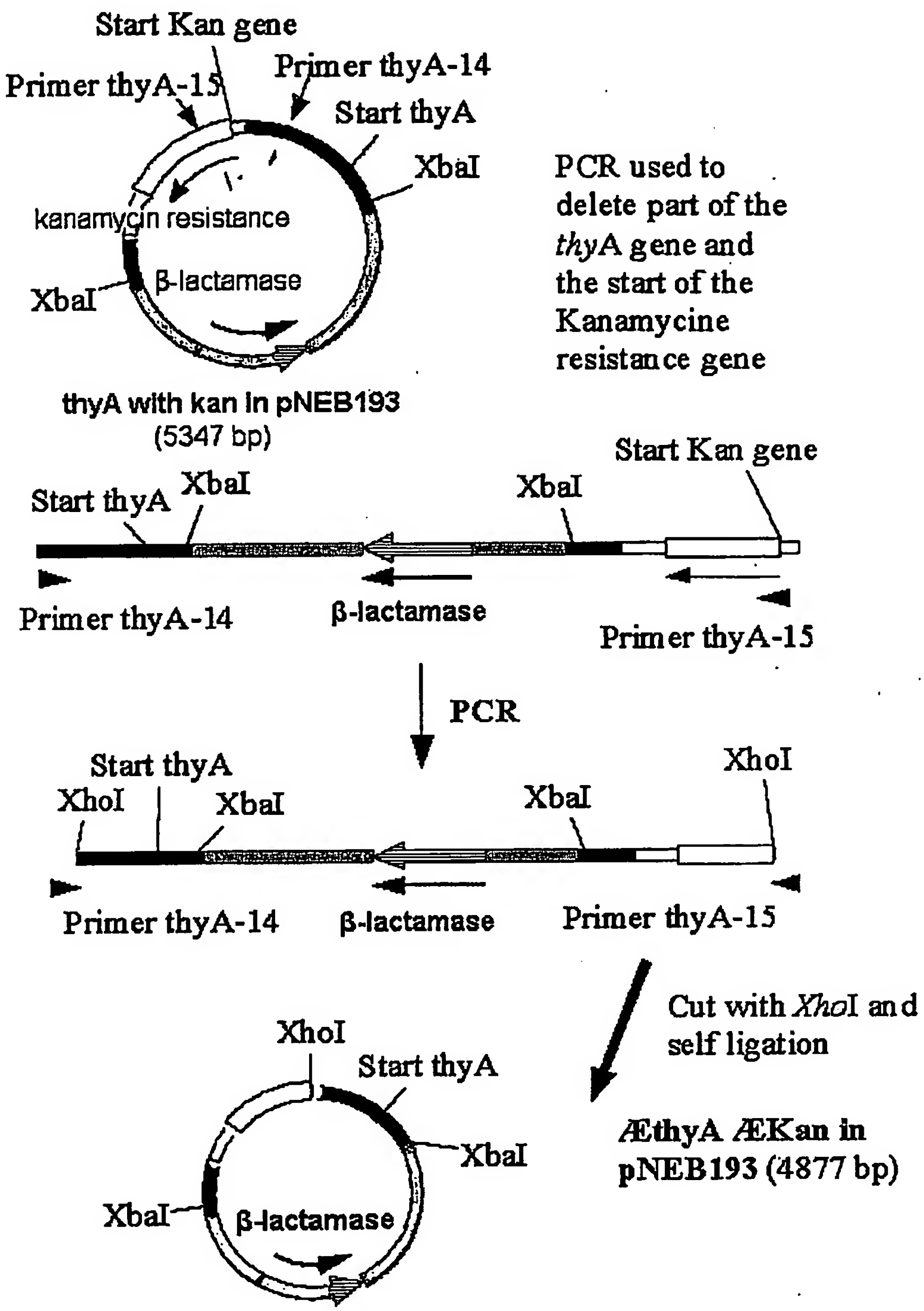
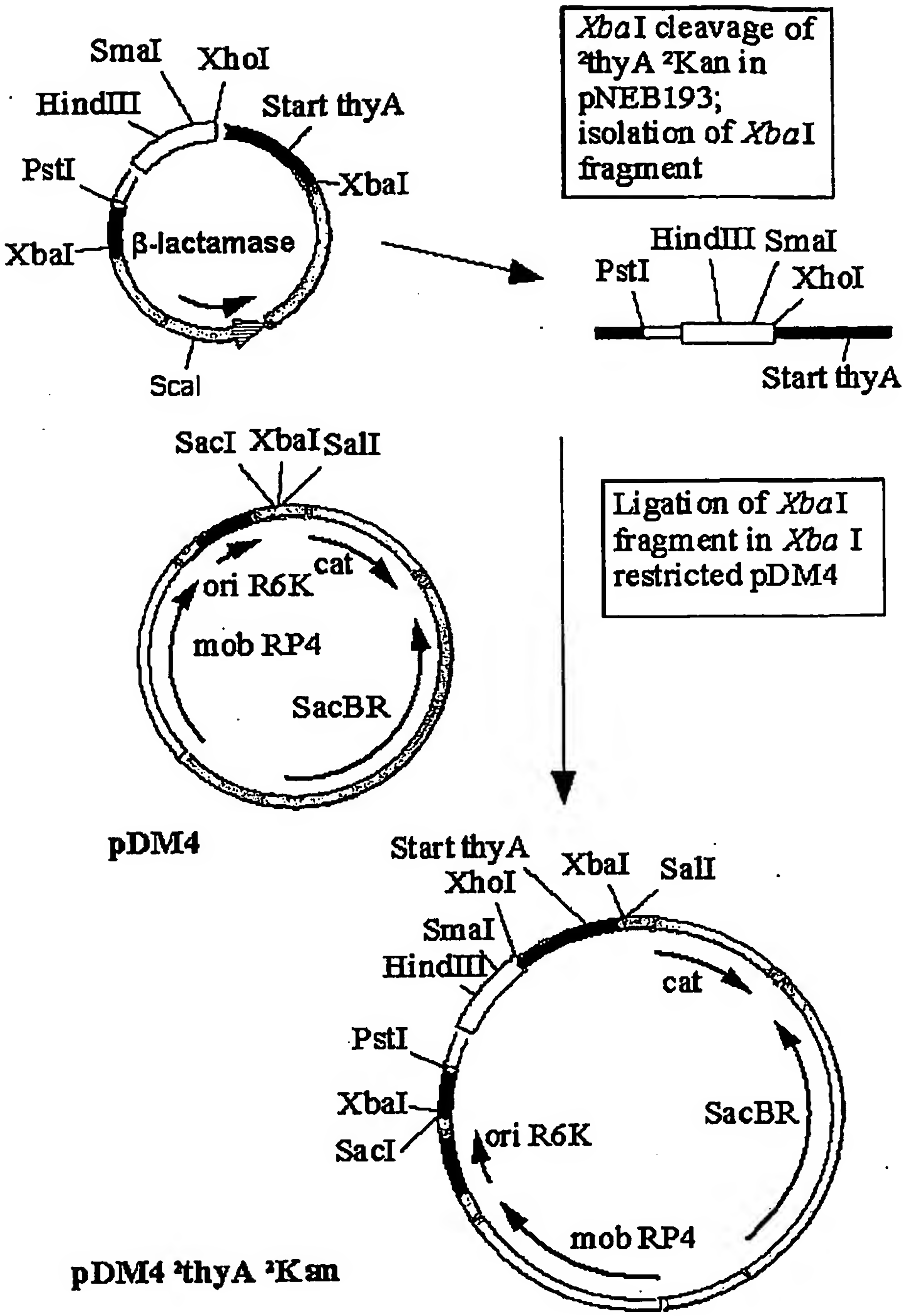

Start Kan gene
Primer thyA-15
Primer thyA-14
Start thyA
XbaI
kanamycin resistance
XbaI
β-lactamase
thyA with kan in pNEB193
(5347 bp)
PCR used to delete part of the *thy*A gene and the start of the Kanamycine resistance gene
Start Kan gene
Start thyA
XbaI
XbaI
Primer thyA-14
β-lactamase
Primer thyA-15
PCR
Start thyA
XhoI
XbaI
XbaI
XhoI
Primer thyA-14
β-lactamase
Primer thyA-15
Cut with *Xho*I and self ligation
XhoI
Start thyA
XbaI
XbaI
β-lactamase
ÆthyA ÆKan in pNEB193 (4877 bp)

Fig. 5



SmaI
XhoI
HindIII
Start thyA
PstI
XbaI
XbaI
β-lactamase
ScaI
XbaI cleavage of ΔthyA ΔKan in pNEB193; isolation of XbaI fragment
HindIII SmaI
PstI
XhoI
Start thyA
SacI XbaI SalI
cat
ori R6K
mob RP4
SacBR
Ligation of XbaI fragment in Xba I restricted pDM4
pDM4
Start thyA
XhoI
XbaI
SalI
SmaI
HindIII
cat
PstI
XbaI
SacI
SacBR
ori R6K
mob RP4
pDM4 ΔthyA ΔKan

Fig. 6

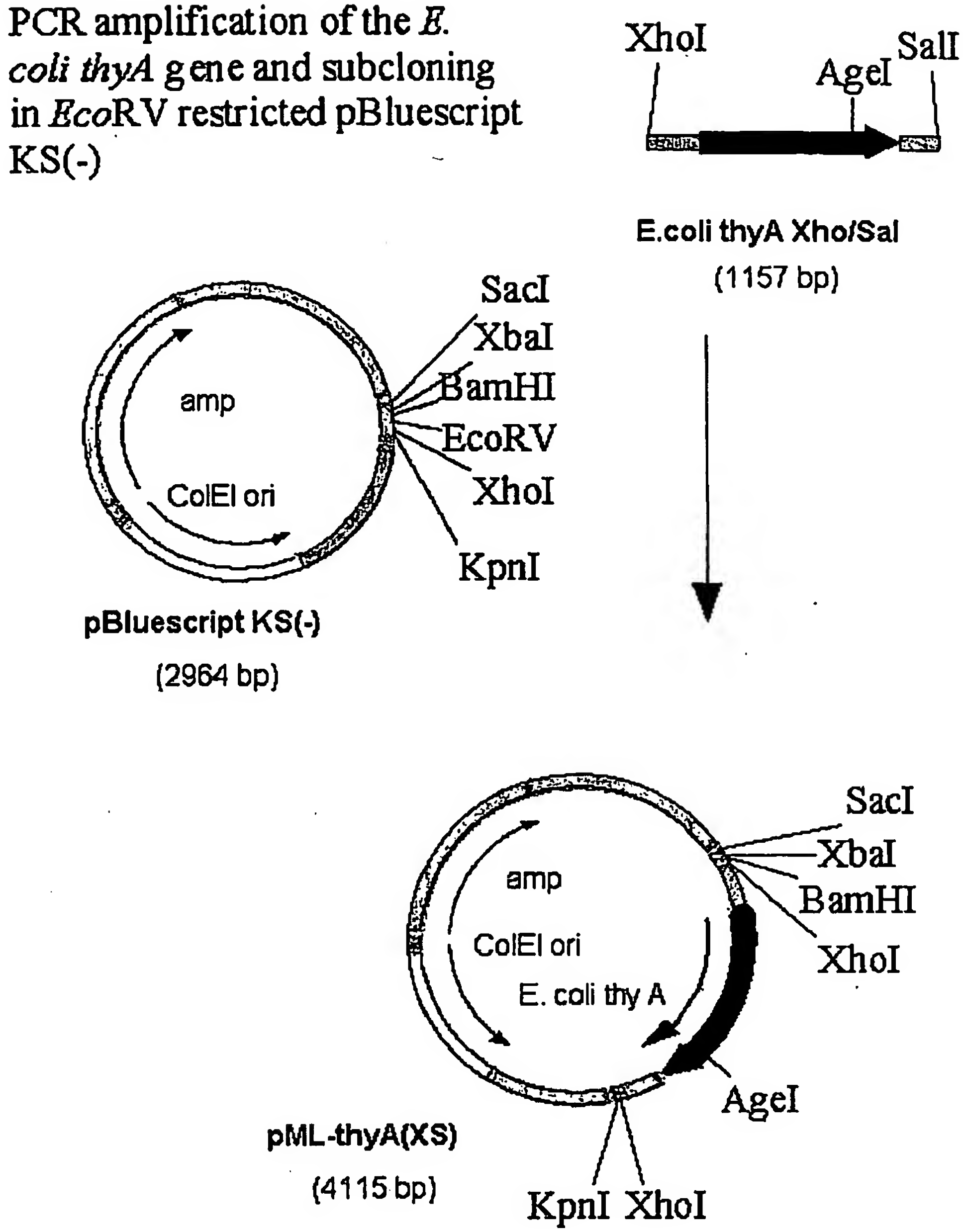

PCR amplification of the *E. coli thyA* gene and subcloning in *Eco*RV restricted pBluescript KS(-)
XhoI
AgeI
SalI
E.coli thyA Xho/Sal
(1157 bp)
SacI
XbaI
BamHI
EcoRV
XhoI
KpnI
amp
ColEI ori
pBluescript KS(-)
(2964 bp)
SacI
XbaI
BamHI
XhoI
amp
ColEI ori
E. coli thy A
AgeI
KpnI
XhoI
pML-thyA(XS)
(4115 bp)

Fig. 7

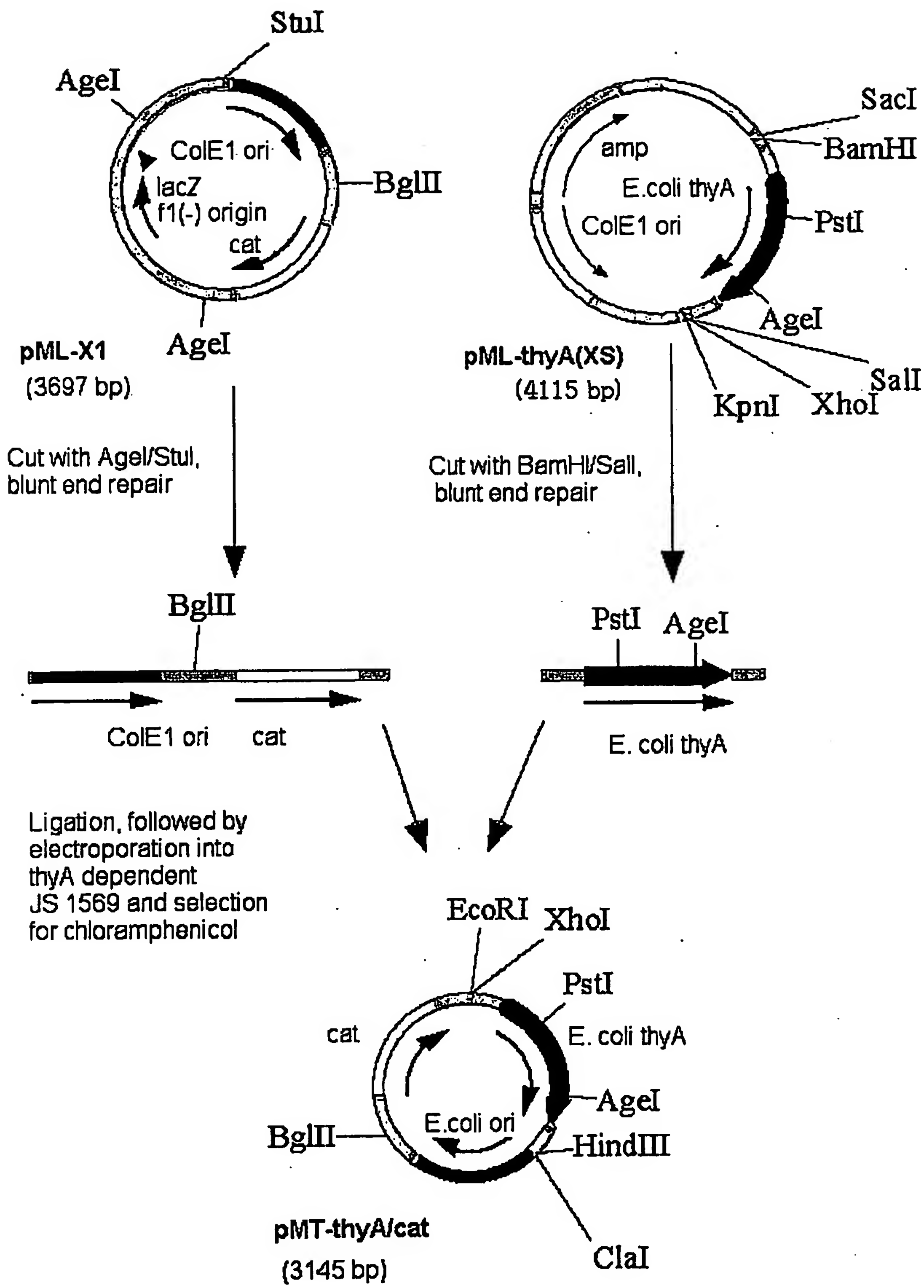

StuI
AgeI
ColE1 ori
lacZ
f1(-) origin
cat
BglII
AgeI
pML-X1
(3697 bp)
SacI
BamHI
amp
E.coli thyA
ColE1 ori
PstI
AgeI
pML-thyA(XS)
(4115 bp)
KpnI
XhoI
SalI
Cut with AgeI/StuI,
blunt end repair
Cut with BamHI/SalI,
blunt end repair
BglII
ColE1 ori
cat
PstI
AgeI
E. coli thyA
Ligation, followed by
electroporation into
thyA dependent
JS 1569 and selection
for chloramphenicol
EcoRI
XhoI
PstI
cat
E. coli thyA
AgeI
BglII
E.coli ori
HindIII
pMT-thyA/cat
(3145 bp)
ClaI

Fig. 8

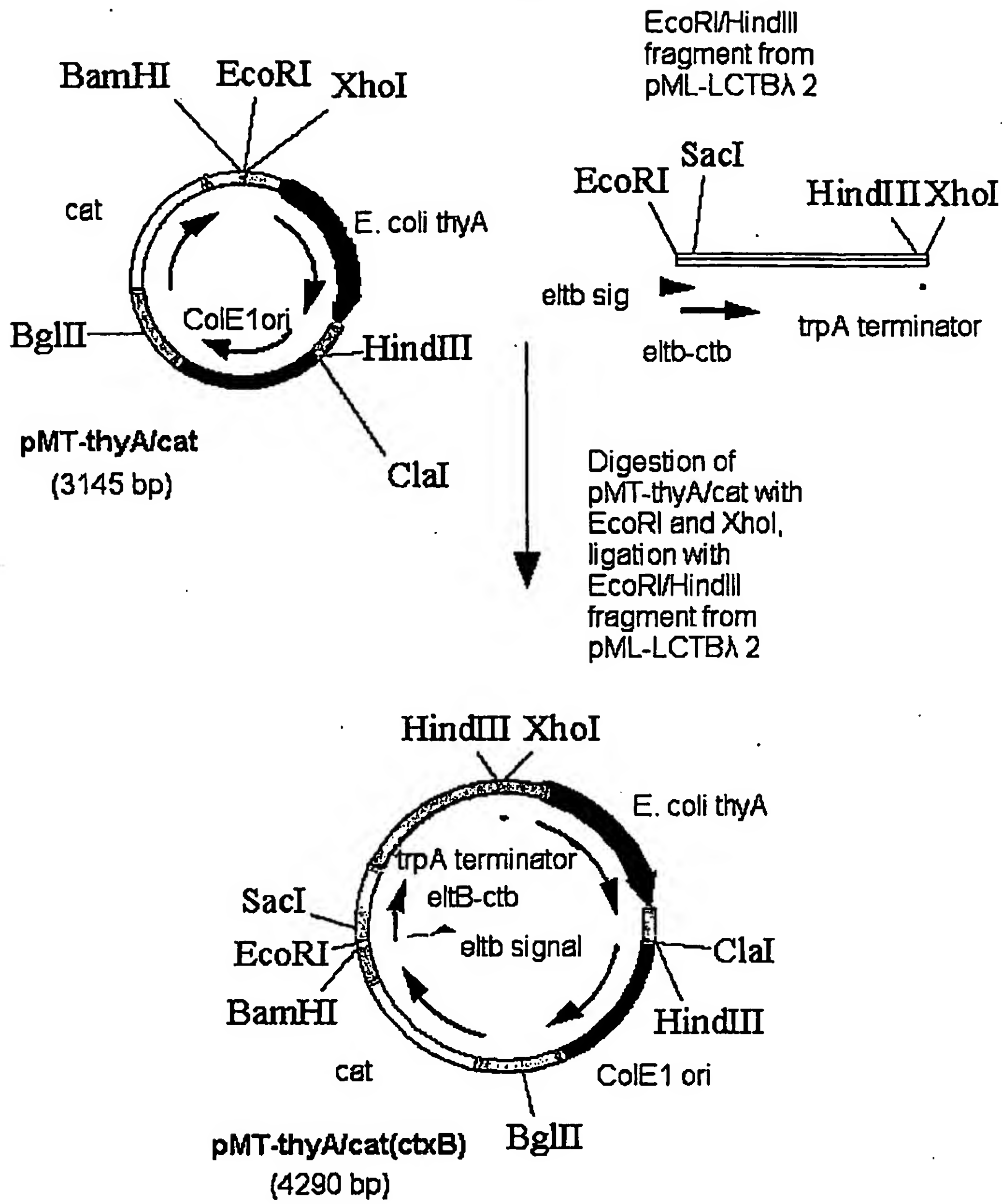

BamHI
EcoRI
XhoI
cat
E. coli thyA
ColE1ori
BglII
HindIII
ClaI
pMT-thyA/cat
(3145 bp)
EcoRI/HindIII fragment from pML-LCTBλ 2
EcoRI
SacI
HindIII XhoI
eltb sig
eltb-ctb
trpA terminator
Digestion of pMT-thyA/cat with EcoRI and XhoI, ligation with EcoRI/HindIII fragment from pML-LCTBλ 2
HindIII XhoI
E. coli thyA
trpA terminator
eltB-ctb
eltb signal
SacI
EcoRI
BamHI
ClaI
HindIII
cat
ColE1 ori
BglII
pMT-thyA/cat(ctxB)
(4290 bp)

Fig. 9

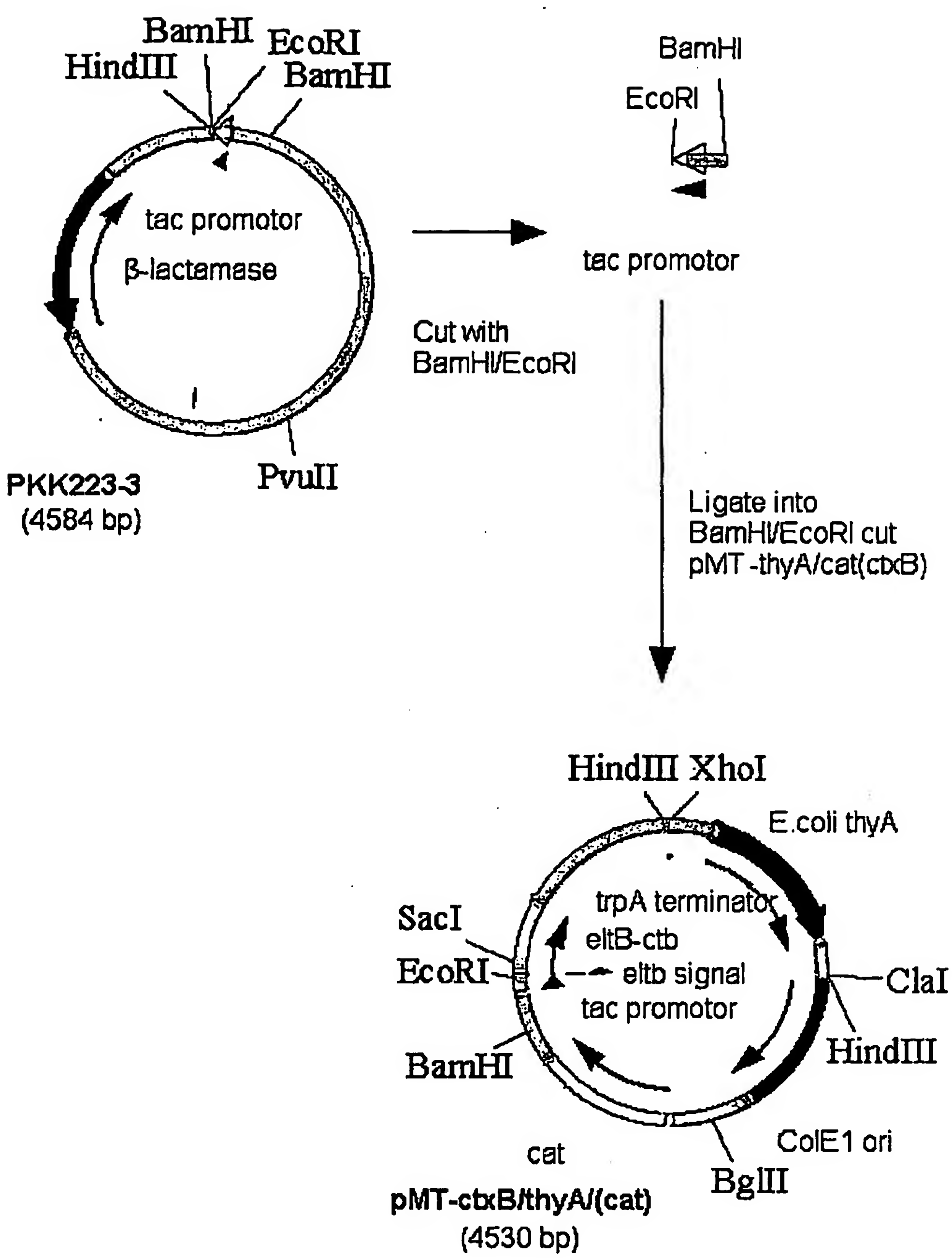

BamHI
EcoRI
HindIII
BamHI
tac promotor
β-lactamase
PvuII
PKK223-3
(4584 bp)
Cut with
BamHI/EcoRI
BamHI
EcoRI
tac promotor
Ligate into
BamHI/EcoRI cut
pMT -thyA/cat(ctxB)
HindIII XhoI
E.coli thyA
trpA terminator
eltB-ctb
eltb signal
tac promotor
SacI
EcoRI
BamHI
ClaI
HindIII
ColE1 ori
BglII
cat
pMT-ctxB/thyA/(cat)
(4530 bp)

Fig. 10

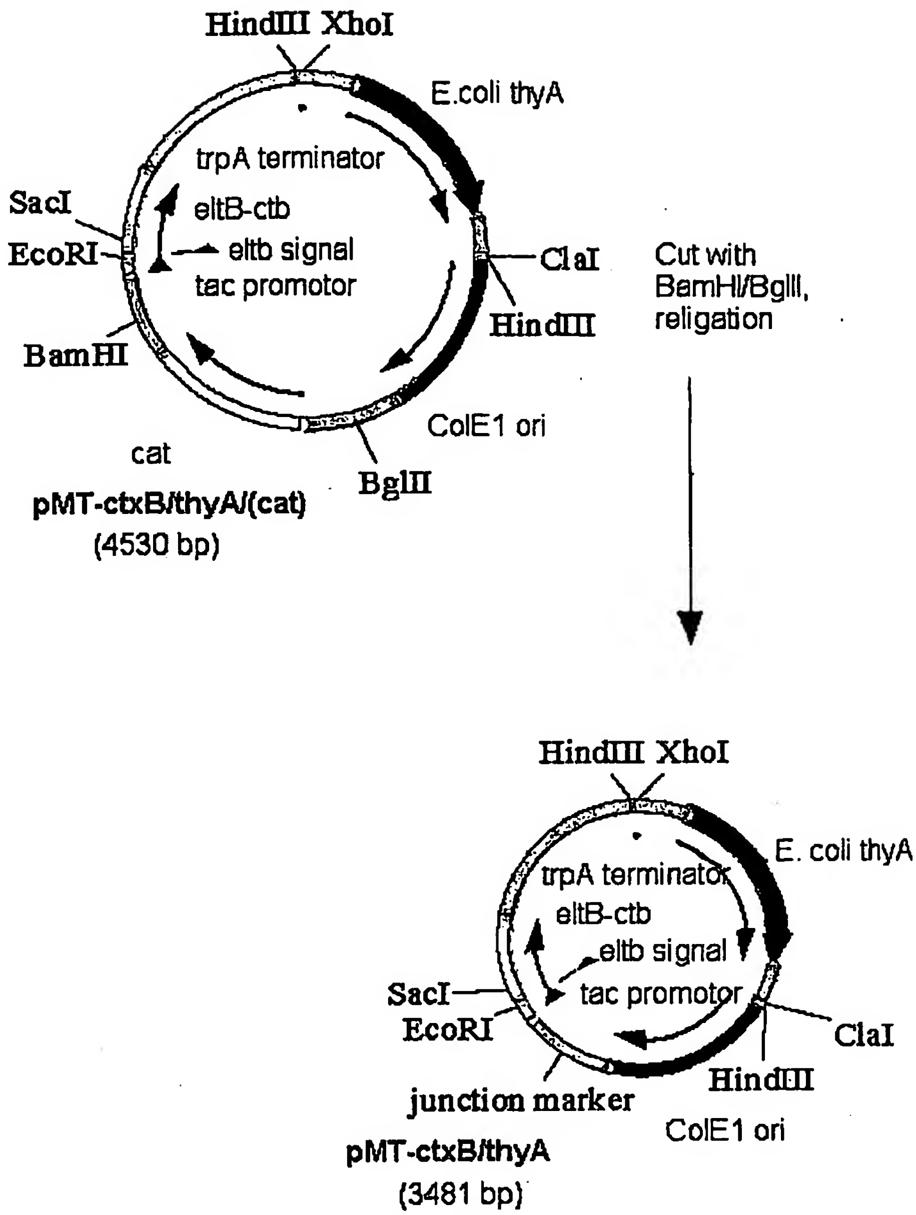

HindIII XhoI
E.coli thyA
trpA terminator
eltB-ctb
eltb signal
tac promotor
SacI
EcoRI
ClaI
HindIII
BamHI
ColE1 ori
cat
BglII
pMT-ctxB/thyA/(cat)
(4530 bp)
Cut with
BamHI/BglII,
religation
HindIII XhoI
E. coli thyA
trpA terminator
eltB-ctb
eltb signal
tac promotor
SacI
EcoRI
ClaI
HindIII
junction marker
ColE1 ori
pMT-ctxB/thyA
(3481 bp)

Fig. 11

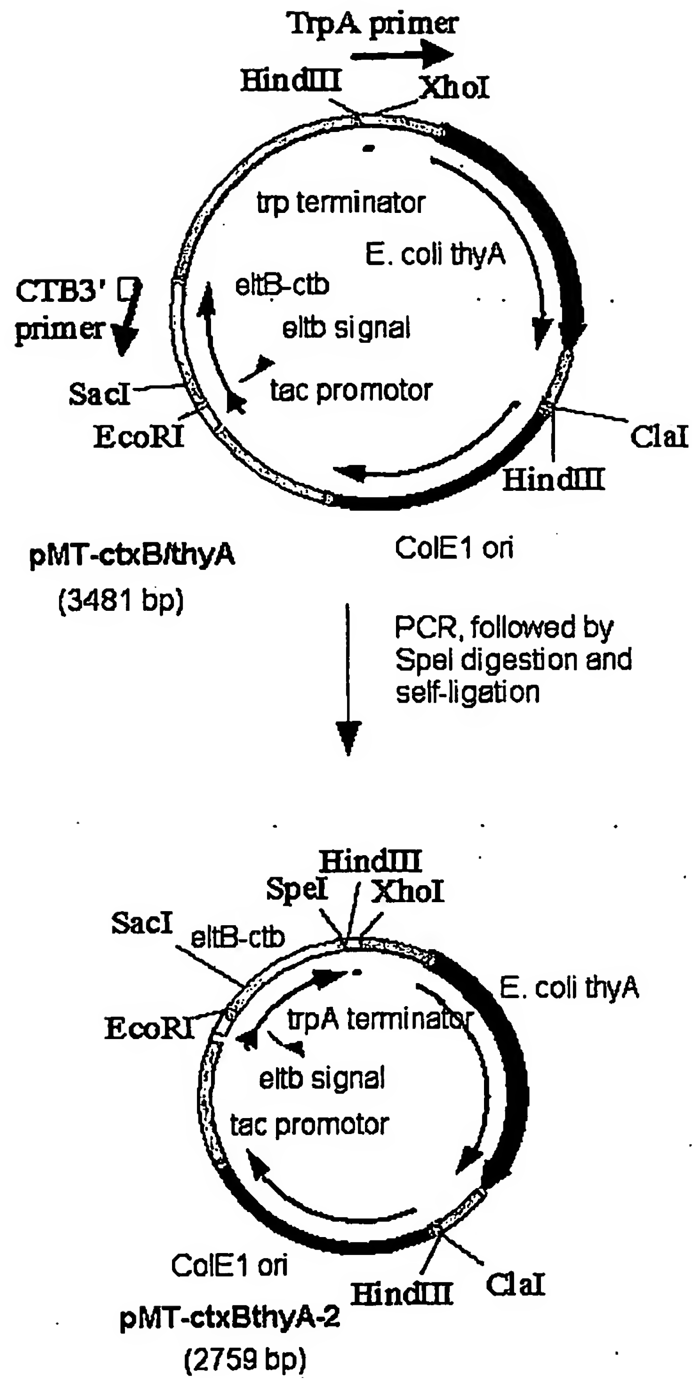

TrpA primer
HindIII
XhoI
trp terminator
E. coli thyA
CTB3' primer
eltB-ctb
eltb signal
tac promotor
SacI
EcoRI
ClaI
HindIII
ColE1 ori
pMT-ctxB/thyA
(3481 bp)
PCR, followed by
SpeI digestion and
self-ligation
HindIII
SpeI
XhoI
SacI
eltB-ctb
E. coli thyA
EcoRI
trpA terminator
eltb signal
tac promotor
ColE1 ori
HindIII
ClaI
pMT-ctxBthyA-2
(2759 bp)

Fig. 12

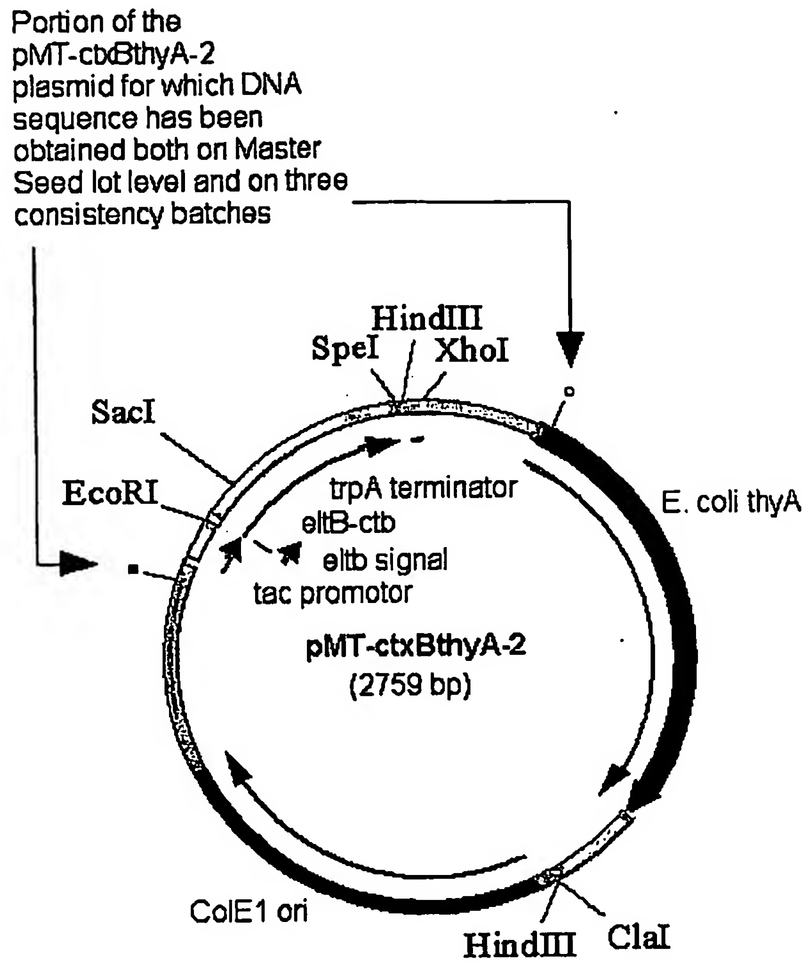

Portion of the pMT-ctxBthyA-2 plasmid for which DNA sequence has been obtained both on Master Seed lot level and on three consistency batches
HindIII
SpeI
XhoI
SacI
EcoRI
trpA terminator
eltB-ctb
eltb signal
tac promotor
E. coli thyA
pMT-ctxBthyA-2
(2759 bp)
ColE1 ori
HindIII
ClaI

Fig. 13

```
          | 10       | 20       | 30       | 40       | 50        | 60       | 70       | 80       | 90       | 100
   1 CTCGAGGTTT GTTCCTGATT GGTTACGGCG CGTTTCGCAT CATTGTTGAG TTTTTCCGCC AGCCCGACGC GCAGTTTACC GGTGCCTGGG TGCAGTACAT 100
 101 CAGCATGGGG CAAATTCTTT CCATCCCGAT GATTGTCGCG GGTGTGATCA TGATGGTCTG GGCATATCGT CGCAGCCCAC AGCAACACGT TTCCTGAGGA 200
        start thyA
 201 ACCATGAAAC AGTATTTAGA ACTGATGCAA AAAGTGCTCG ACGAAGGCAC ACAGAAAAAC GACCGTACCG GAACCGGAAC GCTTTCCATT TTTGGTCATC 300
 301 AGATGCGTTT TAACCTGCAA GATGGATTCC GGCTGGTGAC AACTAAACGT TGCCACCTGC GTTCCATCAT CCATGAACTG CTGTGGTTTC TGCAGGGCGA 400
 401 CACTAACATT GCTTATCTAC ACGAAAACAA TGTCACCATC TGGGACGAAT GGGCCGATGA AAACGGCGAC CTCGGGCCAG TGTATGGTAA ACAGTGGCGC 500
 501 GCCTGGCCAA CGCCAGATGG TCGTCATATT GACCAGATCA CTACGGTACT GAACCAGCTG AAAAACGACC CGGATTCGCG CCGCATTATT GTTTCAGCGT 600
 601 GGAACGTAGG CGAACTGGAT AAAATGGCGC TGGCACCGTG CCATGCATTC TTCCAGTTCT ATGTGGCAGA CGGCAAACTC TCTTGCCAGC TTTATCAGCG 700
 701 CTCCTGTGAC GTCTTCCTCG GCCTGCCGTT CAACATTGCC AGCTACGCGT TATTGGTGCA TATGATGGCG CAGCAGTGCG ATCTGGAAGT GGGTGATTTT 800
 801 GTCTGGACCG GTGGCGACAC GCATCTGTAC AGCAACCATA TGGATCAAAC TCATCTGCAA TTAAGCCGCG AACCGCGTCC GCTGCCGAAG TTGATTATCA 900
                                                                                                         end thyA
 901 AACGTAAACC CGAATCCATC TTCGACTACC GTTTCGAAGA CTTTGAGATT GAAGGCTACG ATCCGCATCC GGGCATTAAA GCGCCGGTGG CTATCTAATT 1000
1001 ACGAAACATC CTGCCAGAGC CGACGCCAGT GTGCGTCGGT TTTTTTACCC TCCGTTAAAT TCTTCGAGAC GCCTTCCCGA AATTTTGCAA CGTCCTGCAA 1100
1101 CGGCGTAAAT AGTCCGGAAG ATGCGCCGAA GAAATAGAAA CGTCGAATCA AGCTTATCGA TACCGTCGAC CTTGAAGAGC AAGGATCTAG GTGAAGATCC 1200
1201 TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT 1300
1301 TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA 1400
1401 ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG 1500
1501 CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC 1600
1601 GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC TATGAGAAAG CGCCACGCTT 1700
1701 CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG CATCTTTATA 1800
1801 GTCCTGTCGG GTTTCGCCAC CTCTGACTTG ATCGTCGATT TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT 1900
```

Fig. 14 cont.

Fig. 14 (cont.)

```
1901 ACGGTTCCTG GCCTTTTGCT GGCCTTTTGC TCACATGTTC TTTCCTGCGT  TATCCCCTGA TTCTGTGGAT AACCGTATTA CCGCCTTTGA GTGAGCTGAT 2000
2001 ACCGCTCGCC GCAGCCGAAC GACCGAGCGC AGCGAGTCAG TGAGCGAGGA  AGCGATGGAA GAGCAGATCC GGGCTTATCG ACTGCACGGT GCACCAATGC 2100
2101 TTCTGGCGTC AGGCAGCCAT CGGAAGCTGT GGTATGGCTG TGCAGGTCGT  AAATCACTGC ATAATTCGTG TCGCTCAAGG CGCACTCCCG TTCTGGATAA 2200
2201 TGTTTTTTGC GCCGACATCA TAACGGTTCT GGCAAATATT CTGAAATGAG  CTGTTGACAA TTAATCATCG GCTCGTATAA TGTGTGGAAT TGTGAGCGGA 2300
                                   start eltB
2301 TAACAATTTC ACACAGGAAA CAGAATTCGG GATGAATTAT GAATAAAGTA  AAATTTTATG TTTTATTTAC GGCGTTACTA TCCTCTCTAT GTGCACACGG 2400
start ctxB
2401 AGCTCCTCAA AATATTACTG ATTTGTGTGC AGAATACCAC AACACACAAA  TACATACGCT AAATGATAAG ATATTTTCGT ATACAGAATC TCTAGCTGGA 2500
2501 AAAAGAGAGA TGGCTATCAT TACTTTTAAG AATGGTGCAA CTTTTCAAGT  AGAAGTACCA GGTAGTCAAC ATATAGATTC ACAAAAAAAA GCGATTGAAA 2600
2601 GGATGAAGGA TACCCTGAGG ATTGCATATC TTACTGAAGC TAAAGTCGAA  AAGTTATGTG TATGGAATAA TAAAACGCCT CATGCGATTG CCGCAATTAG 2700
       end ctxB                       trpA terminator
2701 TATGGCAAAT TAAACTAGTC AATTGAAGCT TAGCCCGCCT AATGAGCGGG  CTTTTTTTT                                             2759
          | 10      | 20      | 30      | 40      | 50          | 60      | 70      | 80      | 90      | 100
```

(SEQ ID NO:1)

204-295: *E. coli thyA* coding region
1192-1876: Col E1 origin of replication
2339-2710: *eltB-ctxB* coding region
2402-2710: *ctxB* coding region
2732-2759: *trpA* terminator

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/03509

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/74 C12N15/54 C12N15/31 C12N9/10 C07K14/245
C07K14/28 C07K14/435 A61K39/106 A61K39/108
//C12R1/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12R C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMPRO2 [Online] E.M.B.L. Databases Accession Number: Y17135, 1 May 1998 (1998-05-01) VALLE E ET AL: "Vibrio cholerae thyA gene" XP002118053	9-13
Y	abstract	1-8

X	EP 0 251 579 A (ENTEROVAX RES PTY LTD) 7 January 1988 (1988-01-07) cited in the application	16
Y	column 6, paragraphs 1,2; claims 17,18	1-8

Y	EP 0 406 003 A (UNIV CORK) 2 January 1991 (1991-01-02) claims 12,13	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 October 1999	05. 11. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lonnoy, O

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/03509

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ATTRIDGE SR: "Thymine auxotrophy as an attenuating marker in Vibrio cholerae" MICROB PATHOG, vol. 19, no. 1, July 1995 (1995-07), pages 11-18, XP002118052 abstract; table 1	16
P,X	DATABASE EMPRO2 [Online] E.M.B.L. Databases Accession Number: AJ006514, 5 June 1998 (1998-06-05) CARLIN N: "Vibrio cholerae lgt and thyA genes" XP002118055 abstract	9-15
P,X	DATABASE EMPRO2 [Online] E.M.B.L. Databases Accession Number: AJ010968, 21 September 1998 (1998-09-21) CARLIN N ET AL: "Vibrio cholerae nptA gene" XP002118054 100% identity in 1187bp overlap with SeqIdNo.3 abstract	11
E	WO 99 35271 A (CAMPOS GOMEZ JAVIER ;LEDON PEREZ TALENA YAMILE (CU); RODRIGUEZ GON) 15 July 1999 (1999-07-15) 99.3% identity in 851bp of Seq.Id.No.1 / 99.3% identity in 283aa of Seq.Id.No.4 claims 9,10,18,19; example 2	1,2,9, 12,13,16
A	WO 94 19482 A (GEN HOSPITAL CORP ;HARVARD COLLEGE (US)) 1 September 1994 (1994-09-01)	
A	US 5 470 729 A (KAPER JAMES B ET AL) 28 November 1995 (1995-11-28)	

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int   .tional application No.
PCT/EP 99/03509

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-9, 12, 13 and 16

A method of producing a Thy-A-deficient strain of Vibrio cholerae comprising the step of site-directed mutagenesis at the locus of the ThyA gene having the nucleotide sequence SeqIdNo:1; A vibrio cholerae thyA-deficient strain lacking the functionality of the thyA gene; said strain comprising one or several episomal autonomously replicating DNA elements having a functional thyA gene that enables the strain to grow in the absence of thymine in the growth medium; said strain wherein said element is a plasmid; said strain comprising a foreign thyA gene; said strain wherein the foreign thyA gene is an E.coli gene; said strain wherein said element also comprises a structural gene encoding a homologous or heterologous protein; said strain wherein said protein is selected from LTB and GST26; A nucleotide sequence and encoded protein of a thyA gene of Vibrio cholerae having essentially the nucleotide sequence SeqIdNo:1; said Vibrio cholerae Thy-A protein having the amino acid sequence SeqIdNo:4; a vaccine comprising as immunising component said thyA-deficient Vibrio cholerae strain.

2. Claims: 10, 14 and 15

A nucleotide sequence of a 5' flanking region of a structural thyA of Vibrio cholerae having the nucleotide sequence SeqIdNo:2; A protein encoded by said nucleotide sequence; said protein having the amino acid sequence of SeqIdNo:5

3. Claim : 11

A nucleotide sequence of a 3' flanking region of a structural thyA of Vibrio cholerae having the nucleotide sequence SeqIdNo:3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/03509

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
EP 0251579	A	07-01-1988	AU	594161	B	01-03-1990
			DK	318887	A	25-12-1987
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